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Genetic Diversity and Camptothecin Variation in Camptotheca Decaisne

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GENETIC DIVERSITY AND CAMPTOTHECIN VARIATION IN *CAMPTOTHECA* DECAISNE

by

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GENETIC DIVERSITY AND CAMPTOTHECIN VARIATION

IN CAMPTOTHECA DECAISNE

by

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ABSTRACT

Camptotheca Decaisne is the major source of the promising anti-cancer alkaloids camptothecins. The main objectives of this study are (1) to reveal the genetic diversity of Camptotheca and to identify taxa with RAPD markers; (2) to determine the camptothecin (CPT) variations within and among different populations and taxa, with tissues, season, and age; and (3) to determine the effects of environmental stress on the camptothecin production in Camptotheca.

RAPD markers provide a powerful tool for the identification of some populations (particularly cultivars) and the detection of genetic variation within Camptotheca. Three primers (OPA02, OPA03, and OPA04), generating 44 polymorphic bands using randomly amplified polymorphic DNA (RAPD) markers, were able to discriminate among 25 Camptotheca populations. The band size varied from 268-4,411bp, with an average of 15 bands/primer. Of these populations, 'Katie', HJ population of C. acuminata, and 'Ang' can be easily distinguished by their unique bands.

Population differentiation of Camptotheca was found to be higher than in other species with similar breeding systems. All populations were therefore genetically distinctive and each should be considered as a management unit. The high level of genetic structure among populations indicates differentiation due to founder events and/or genetic drift coupled with limited migration.

Cluster analysis of the genetic distance values and dendrogram from RAPD markers are consistent with the phenotypic data and both support the current taxonomic treatment of Camptotheca. Camptotheca acuminata var. acuminata appeared as the closest relative of C. yunnanensis, followed at some distance by C. lowreyana and, further away, C. acuminata var. tenuifolia. Strong interspecific crossing barriers exist between C. lowreyana and C. yunnanensis due to geographical isolation.

The CPT data of Camptotheca are consistent with the phenotypic and genetic variation analysis. The results show that CPT variation in Camptotheca is mainly determined genetically under the same undisturbed growth conditions. Variation in leaf CPT content of Camptotheca is greater among species than within species. Camptotheca acuminata has relatively low CPT contents and less variation among populations. Considering both low genetic diversity and low CPT yield, the species is not the optimum candidate for plantation development for CPT production. In contract, C. lowreyana should be considered as a management target in both CPT production and germplasm conservation because the species not only has higher genetic diversity but also has higher CPT concentrations than the other taxa. Young photosynthetic leaves and stems have higher CPT contents than old ones, but 'sink' tissues such as woods, roots, and fruits show different patterns. CPT content also shows a great seasonal change, but it is less influenced by tree age.

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INTRODUCTION

Camptotheca Decaisne is a Chinese genus of the family Nyssaceae. It is the major natural source of camptothecin (CPT), an anti-cancer alkaloid. CPT and its analogs have shown promising anti-cancer activity against many kinds of cancers in clinical trials in the USA, China, Japan, and Europe beginning in 1957, particularly since 1986 (Li and Adair, 1994). The CPT agents have also shown potent anti-viral activity against HIV in both animal and human cell cultures (Priel et al., 1993). In 1996, Topotecan (TPT, Hycamtin®) and Irinotecan (CPT-11, Camptosar®), two semi-synthetic CPT drugs, were approved by the FDA for the treatment of patients with advanced ovarian and colon cancers, respectively. In 1998, Topotecan was approved by the FDA for the treatment of small cell lung cancer. Another CPT analog, 9-Nitrocamptothecin (9-NC, Brutecan®), which has received a response rate of over 50% in pancreatic cancer clinical trials, will also be approved by the FDA soon (Stehlin Foundation, pers. comm., 1999). The compound 9-Aminocamptothecin (9-AC) and several other CPT analogs (e.g., CZ112) have also shown promising results. In fact, CPT agents have been recognized as the most promising anti-cancer drugs in the world. Therefore, the worldwide demand for CPT is dramatically increasing.

Currently, CPT production is still dependent on natural sources. Camptotheca trees grow fast and many parts of the trees can be used to extract drug CPTs (Li et al., 2000). There are several major problems with the development of Camptotheca as a drug resource. First, the genus is in endangered status in its natural range and may be nearing extinction in the immediate future. Second, the gene pool of Camptotheca in the USA is extremely small. Most of the trees in this country are traceable to two mature trees in Chico, California that germinated from seeds imported from southern China in 1934 (Li and Adair 1994). Selfing is often the only breeding system for these plants and the offspring are normally of low quality. Also, cold-hardiness and drought-tolerance are two major problems in plantation development in the southeastern USA. However, the present genetic resource base of Camptotheca in the USA is too small to select frost- and drought-tolerant and high-CPT-yield genotypes.

Studies on Camptotheca trees were started in the early 1990s with support from the Houston Livestock Show and Rodeo and Fondren Foundation. Researchers in the SFA College of Forestry have conducted a three-phase study on Camptotheca (Li and Adair 1994; Li 1997; Li et al., 2000). Phase I of the study is the evaluation and forecast of CPT agents as anti-cancer and anti-viral drugs. Phase II of the study is the worldwide resource investigation of Camptotheca including description of a new species, identification of endangered

status in nature, and database construction. Phase III of the study is the development of strategies to maximize CPT production in trees. This study includes establishment of an exclusive germplasm preserve, development of a high-CPT-yield cultivar, discovery of the CPT accumulation sites in trees, induced production of CPT in plants, and improvement of CPT extraction methods. Also, some physiological studies of Camptotheca have been conducted at Texas A & M University and Louisiana State University (Lopez-Meyer et al., 1994; Jain and Nessler 1996; Liu and Adams 1996, Lineberger et al., pers. comm., 1997). Obviously, however, there is no systematic study on the genetic diversity and CPT production ecology of Camptotheca although such a study is imperative to conservation and improvement of these endangered, valuable species and enhancement of CPT production in the trees.

The main objectives of this research are:

1. To reveal the genetic diversity of Camptotheca and to identify species/varieties/cultivars with RAPD markers.
2. To determine the camptothecin variations within and among different populations (species, varieties, or cultivars), tissues, seasons, and ages.
3. To determine the effects of environmental stress on camptothecin production in Camptotheca.

LITERATURE REVIEW

The earliest literature on Camptotheca appeared in 1848. In his classical Chinese botanical book, Wu (1848) briefly described the morphology and habitat, but not uses of the tree. However, there were only limited studies on Camptotheca until the early 1950s. Research interests have been dramatically increasing since 1957 when the anti-cancer activity of the tree was discovered, particularly after the mechanism of action of CPTs was found in 1985. To date, there are about 15,000 publications on Camptotheca and CPTs, with 80% of these published in the last 15 years. More than 90% of these studies were in the fields of chemistry, pharmacology and clinical trials of CPTs, with very little research focused on the biology and ecology of Camptotheca.

In 1994, "Camptotheca acuminata Decaisne, Xi Shu, Chinese Happytree, a Promising Anti-tumor and Anti-viral Tree for the 21st Century" (Li and Adair 1994), the first monograph on Camptotheca and CPTs, was published by the SFA College of Forestry. The book outlines different aspects of C. acuminata. The first part of the book covers the history of CPT discovery, comparisons with taxols, mechanisms of action, preclinical and clinical trials in cancer treatment, anti-viral activity, other uses, and drug sources; the second part involves botany, geography, ecology, reproduction, growth, protection, harvest, and further research of the trees; and the third part is a bibliography of over 1,300 citations

worldwide. The second monograph by Li, which will mainly present recent research results on Camptotheca at SFA, soon will be published.

The biochemical and clinical studies of CPTs have been reviewed in detail (Slichenmyer et al., 1993; Li and Adair 1994, Tanizawa et al., 1994; Liu et al., 1997; Robert and Rivory 1998). Therefore, this literature review will focus on botanical, ecological, and CPT yield studies of Camptotheca.

Taxonomic Treatments

The genus Camptotheca Decaisne was established based on specimens of C. acuminata by J. Decaisne in 1873. The type specimen was collected by Father A. David in Lushan, Jiangxi Province, during his 1868-1870 exploration of China. For over a century, C. acuminata was the only species recognized in the genus. Two varieties, var. tenuifolia Fang et Soong and var. rotundifolia Yang et Duan were described in 1975 and 1988, respectively (Fang and Soong 1975; Yang and Duan 1988). Because either the type tree or the type specimen was destroyed and no other collections were made after the publications, the present authors recognized these two varieties according to the original description and single type specimen (var. tenuifolia) or original description only (var. rotundifolia).

Camptotheca yunnanensis was described by A. Dode in 1908 based on the specimen collected by Delavay (at the Herbarium of the Museum of Paris) in

September 1888 from Yunnan. Dode emphasized that C. yunnanensis had smaller fruit (1.5 cm long). But Wilson (1914) believed that Dode's type specimen had immature fruits only because it was collected in September. C. yunnanensis has never been studied because of its incomplete original description with no further collections. Wilson (1914) simply treated C. yunnanensis as a synonym because he believed that the characteristics of C. yunnanensis were within the variation range of C. acuminata (Li and Adair 1994). Recently, Li conducted a monographic study of the genus (Li and Adair 1994; Li 1997; Li et al., 2000). Based on field observation and phenotypic and ecological analysis in both mature and juvenile stages, Li recognized C. yunnanensis as a species separate from C. acuminata (Li 1997). Naturally, C. yunnanensis is restricted to the tropical forests in Yunnan, China. The species has been cultivated as a street tree in several places in Yunnan, where it has been commonly recognized as C. acuminata. Actually, C. yunnanensis is significantly distinguished from other taxa in Camptotheca by its (1) semi-deciduous, elliptic leaves with 12-15 lateral veins on each side, (2) gray, smooth, lucid, thin three-winged fruits, and (3) red hypocotyl (before primary leaf appears) and linear, pinnipalmate cotyledons.

More recently, C. lowreyana Li, the third species, was described in honor of the late L. Lowrey, a Texas horticulturist (Li 1997). This species differs from existing taxa by its cordate or ovate leaves with greenish and lucid lower

surfaces, gray-brown, smooth, lucid, and longer fruit, and pinninerved cotyledons with more lateral veins on each side.

To date, therefore, this previously monotypic genus includes: C. acuminata (var. acuminata, var. rotundifolia, and var. tenuifolia), C. yunnanensis, and C. lowreyana, (var. lowreyana, cultivar 'Katie', cultivar 'Hicksii', and cultivar 'Ang') (Li et al., 2000; Li 2001).

Morphology

Morphological data on Camptotheca are limited. Recently, an analysis in phenotypic variation within and among species was made by Li et al. (unpublished). Eighteen characters have been measured for their morphometric analysis among populations: bark feature, leaf vein number, leaf blade ratio (width/length), leaf shape, bract shape, bract pubescence, calyx shape, calyx pubescence, petal shape, petal pubescence, fruit length, fruit width, fruit color, fruit surface texture, cotyledon vein number, cotyledon ratio (width/length), pollen size, and pollen surface texture. Interestingly, fruit color and surface texture were quantitatively analyzed by image processing technology as a new application in botanical study. The authors provided much valuable new data on morphological variations within different generations, developmental stages, growing conditions, populations and species.

Leaf micromorphology provides important quantitative and qualitative traits in Camptotheca. Stoma density and size (length and width), subsidiary cell number, and gland length on lower leaf surfaces in Camptotheca were studied using light microscopy and scanning electron microscopy (SEM) (Li et al., unpublished). Like Nyssa (Metcalfe and Chalk, 1957), Camptotheca has simple unbranched non-glandular hairs, external unicellular glands, and stomata on the lower surface of leaves (hypostomatous). Stoma size and frequency, outer stomatal rim, subsidiary cell number, and gland shape and size display diagnostic values, particularly at the species level.

Ethnobotany

In China, the popular common name for Camptotheca is Xi Shu ("happytrees"). It is not clear when the name came into use. But it is named as such because the tree can be used as a folk medicine to cure stubborn phlegm as well as other diseases and thus make patients happy (Ran 1993). Here the stubborn phlegm refers to the phlegm syndrome which is difficult to cure, and which is the cause or manifestation of stubborn diseases, such as recurrent asthma, and headache. Xi Shu was translated into English as "happytree" (Li and Adair 1994). Currently, C. acuminata var. acuminata is widely known as the Chinese happytree in the English-speaking world, but it is also known as the "Tree of Joy" in Louisiana, USA. However, the English name "happytree" is more

appropriate to its original Chinese meanings. In China, more than 50 local names are available for Camptotheca and most refer to tree morphology, habitat, and uses (Li et al., unpublished).

It is widely recognized that Camptotheca had little human use in its native region of China before the 1960s (Perdue et al., 1970). The discovery of its anti-tumor activity has now made it the "Cinderella of the forest" (Li and Adair 1994). There was no scientific evidence of the use of Camptotheca for medicinal purposes either within or outside China before 1958 when the anti-tumor activity was discovered by chemists (M. E. Wall, pers. comm., 1995). Recently, however, it was found that Chinese Dong people have been using Camptotheca for traditional drug purposes for hundreds and possibly thousands of years (Li, unpublished). This new finding of ethnic use may lead to new directions for medicinal studies of the species. It also validates the importance of ethnobotanical studies in the utilization of plant resources (Cox and Balick 1994).

Ecology

Camptotheca is a Tertiary relict genus. Fossils of the genus were recorded in several locations in Japan from the Tertiary period (Suzuki 1976; Tanai 1977), and relatives of Camptotheca were recently reported in Paleocene floras of the Rocky Mountains and Great Plains in North America (Manchester 1997). At present, the genus is naturally restricted to southern China.

According to recent field investigations (Li et al., 2000), all three species of Camptotheca are in endangered status under natural conditions and may be nearing extinction in the immediate future. Camptotheca acuminata is naturally restricted to some remote mountainous areas in southern China with a population of less than 5,000 wild trees (Li et al., 2000). One variety, var. rotundifolia had only one known specimen (holotype) which was destroyed a few years ago. Another, var. tenuifolia, had only one tree identified since it was discovered in 1975. This sole tree was removed eight years ago and no more living specimens have been located in the area. Camptotheca lowreyana has only about 50 mature trees in nature. Genetic variation dramatically decreases within populations of Camptotheca because selfing and related matings are common in both natural and cultural conditions. This decline in genetic diversity is exacerbated by current population structures (1-20 trees in a population), distribution patterns (populations isolated from each other), and increasing demand. There is no genetic resource base and no current effort is being made to preserve the dramatically decreased genetic diversity of Camptotheca (Li et al., 2000). Fortunately, the Chinese government has listed Camptotheca spp. as a state endangered species and it is now under protection.

Camptotheca are riparian trees, and naturally grow on well-drained fertile soils in warm and humid subtropical China (Li and Adair 1994).

Camptotheca acuminata has been widely introduced to many gardens and arboreta in North America, Asia, and Europe as living collections since 1934 (Li

and Adair 1994). The worldwide cultivation history of C. acuminata has been reviewed (Li and Adair 1994). Recently, there has been increasing interest in Camptotheca plantation development for CPT extraction in the United States, India, Japan, France, Germany, Australia, and Brazil. The lack of cold-hardiness and drought-tolerance are two main factors limiting the development of the plant resources in these countries. However, the genetic base for plantation resources outside China is too narrow and small in number to allow selection of an ecotype for cold-tolerance, drought-resistance, or high biomass/drug production. In the United States, for example, C. acuminata was successfully introduced in 1934 and today has only about 20,000 seedlings largely in California, Hawaii, Louisiana, South Carolina, and Texas. However, most of these trees are traceable to two mature trees in Chico, California that germinated from seeds imported from southern China in 1934 (Li and Adair 1994; see Figure 1). Thus, relatedness is common within plantation populations due to this limited seed source. Selfing is often the only breeding system for these plants and the offspring are normally of low quality. Solutions to these genetic and adaptability problems are largely dependent on the expansion of the genetic base in China. However, to date little data are available on current resources in China. In fact, such a survey for Camptotheca does not exist.

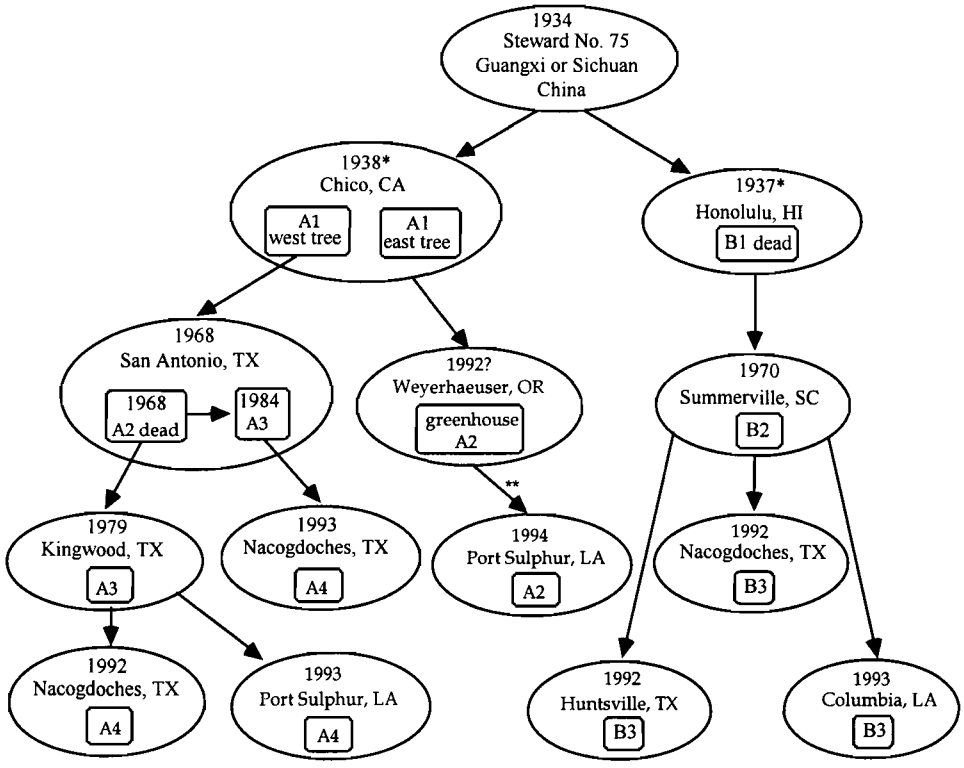


Figure 1. Pedigree of principal seed sources of C. acuminata in the United States (letter with number represents generation number; * seedlings were produced in 1935; ** reproduced by tissue culture, others reproduced by seeds) (Li et al., 2000).

Reproduction

Camptotheca is polygamo-monoecious. The stamens are shed nearly one week before the stigma of the same flower becomes receptive; this protandry leads to cross-pollination as the major breeding system in Camptotheca (Chen 1988; Chen et al., 1991; Li and Adair 1994). Pollination of Camptotheca is obligately entomophilous, and fruit production depends on the activities of pollinating insects (Chen et al., 1991).

It is relatively easy to reproduce seedlings by seed, a common propagation method in Camptotheca. Heating and stratification may increase the germination rates of seeds (Perdue 1968; Perdue et al., 1970; Shao 1989; Zhou 1989).

Camptotheca has great coppice ability and can be propagated vegetatively. Propagation by shoot and leaf cuttings has been studied at SFA and several other universities and nurseries. Micropropogation of C. acuminata by shoot bud, shoot tips, seed embryos, and cotyledon tissue culture has been studied at several universities (Jain and Nessler 1996; Lineberger, pers. comm., 1997; Liu and Li 2001). Recently, tissue culture of other Camptotheca species and varieties has began to be investigated at SFA.

Camptotheca grows rapidly, up to 3 m annually in the juvenile stage under favorable conditions. Trees can start fruiting at 4-5 years of age. Mature trees can reach 45 m in height under natural conditions (Li et al., 2000).

Genetic Diversity

It is important to understand the genetic structure of a population or species for conservation and management strategies. Traditionally, genetic resources have been characterized by a combination of morphological and agronomic traits (Chalmers et al., 1992). In forest management, provenance or geographical variation is often considered as an accurate predictor of the diversity spectrum within a species. This approach has been challenged by many recent studies (Chalmers et al., 1992). Isozymes have been used extensively to monitor genetic diversity for plant species since the 1970s. Since the late 1980s, many authors have used restriction-site diversity to infer population genetic structure (Clegg 1989). Restriction fragment length polymorphisms (RFLP) are the most frequently used type of DNA marker. RFLP analysis requires large quantities of relatively pure DNA and species-specific DNA probes, and is also labor intensive. Polymerase chain reaction (PCR) development revolutionized DNA analysis (Saiki et al., 1988). However, the PCR procedure requires DNA-sequence information.

Recently, Williams et al. (1990) and Welsh and McClelland (1990) developed a novel RAPD (randomly amplified polymorphic DNA) technique for identification of polymorphism in plants based on PCR, which does not require prior DNA sequence information. This technique has provided a powerful tool for the investigation of genetic variation. The RAPD procedure is simpler and less costly

than other DNA marker methodologies and requires very small amounts of DNA. RAPD markers have been successfully used in identification and classification of plants, e.g., crops (Williams et al., 1990; Klein-Lankhorst et al., 1991; Welsh et al., 1991; Wilde et al., 1992; Yang and Quiros 1993), ornamental species (Arnold et al., 1991; Carlson et al., 1991; Kamalay and Carey 1995; Lqbal et al., 1995; Gawel et al., 1996), rare species (Brauner et al., 1992), forage species (Huff et al., 1993), and nitrogen-fixing species (Chalmers et al., 1992). These studies have shown that inbred plants usually have extensive RAPD divergence among, but little variation within, species or populations. In contrast, outcrossing plants have considerable RAPD variation within species or populations (Brauner et al., 1992; Huff et al., 1993). The RAPD markers will provide an important means to identify the species/clones of Camptotheca, particularly in the early stages of plant development, since it is difficult to distinguish the species/varieties prior to leaf formation.

CPT Yields

Presently, CPT production still relies on extractions from Camptotheca. The existing studies on CPT yield in Camptotheca by other authors are contradictory, and all are limited to C. acuminata, since the plant materials of other species and varieties are not widely available. Most studies showed that almost all parts of C. acuminata could yield CPTs with concentrations ranging from 0.004% to 0.400% of dry weight (Li and Adair 1994). Hsu and coworkers

found that the content of CPTs in different parts of C. acuminata are at an average rate of 5:10:5:2:15 for roots:root bark:stem bark:stems:fruits (Hsu et al.,1977a, b). Fruits have the highest yield of CPTs according to these authors. This view has been widely accepted and thus fruits are commonly used for CPT extraction in China (Li et al., 2000).

As early as 1957, leaves were reported to have anti-tumor activity (Wall et al., 1966), indicating that leaves contain anti-tumor CPTs. Later, however, little and even no CPTs were reported in leaves by some researchers (Perdue et al., 1970). In 1996, Liu and Adams also reported no significant difference between young and old tissues in C. acuminata. Some other authors, in contrast, stated that leaves had a higher CPT concentration (0.040%) than either roots (0.036%) or stems (0.016%) (on dry-weight basis) (Tien et al., 1977), and that leaves contained enough toxic CPTs to kill goats feeding on them (Cao et al., 1992). However, these authors did not describe what kind of leaf materials (e.g., leaf age, or location on the trees, or harvest season) were used in their experiments. In 1994, it was found that Chinese Tung people in Guangdong Province had been using extracts of young leaves of Camptotheca with alcohol as the solvent for stubborn skin diseases (e.g., skin cancers) for hundreds of years (Li et al., 2000). Lopez-Meyer and his colleagues (1994) found that in C. acuminata, young leaves rather than old ones produced up to 0.40% of CPT (on dry-weight basis), approximately 50% higher than in fruits and 250% higher than in the bark. More recently, Liu and his colleagues amended their previous 1996 results and

stated that young leaves had higher CPT concentration than old ones (Liu et al., 1998, 1999). The contradictory results from the authors are probably largely caused by sampling and analysis problems (e.g., experimental materials, sample collection, size, and time, and extraction methods). To date, it is widely accepted that young leaves of C. acuminata have higher CPT than old ones although other Camptotheca taxa have not been investigated. Even if true in all Camptotheca species/varieties, the basis for this phenomenon has not been explained.

Roles and Biosynthesis of CPTs

Knowledge of accumulation sites and distribution patterns of CPTs in Camptotheca seems critical to understanding the CPT biosynthesis pathway, tree improvement, and CPT production. CPTs are a family of alkaloids, which are secondary metabolites restricted to plants. Previously, it was believed that secondary metabolites have little explicit function in plants. Recently, however, evidence indicates that numerous secondary products play a physiological and ecological role in plants (Shimomura et al., 1997). Our recent analysis showed that there is a positive relationship between the density and volume of glandular trichomes and CPT content regardless of species (Li et al, unpublished data, Figure 2). Fluorescence and scanning electron microscopic studies have shown that CPTs are highly concentrated in trichomes at the early stage of plant organ development. It is known that CPTs are cytotoxic to both human and animals as well as insects (Li & Adair 1994). Therefore, the trichomes in Camptotheca could

play a defensive role against herbivory (Li et al., unpublished data). This finding, along with the previous studies on other plants such as Nicotiana and Solanum (Levin 1973; McKey 1979; Tingey and Laubengayer 1981; Van Dam et al., 1994) suggests that glandular trichomes in higher plants may commonly produce secondary metabolites (e.g., alkaloids) to defend against insect herbivory and microbe attacks.

In addition, CPTs may have physiological roles in plant development (Li et al., unpublished data). CPTs are known to inhibit the Topo I enzyme and thus block vigorous cell division in humans and animals (cancers) (Hertzberg et al., 1989). Similarly, in Camptotheca plants or cell culture systems CPTs may have the same effect. In other words, biosynthesized CPTs inhibit the Topo I enzyme through binding, thus becoming an inhibiting factor to regulate plant growth.

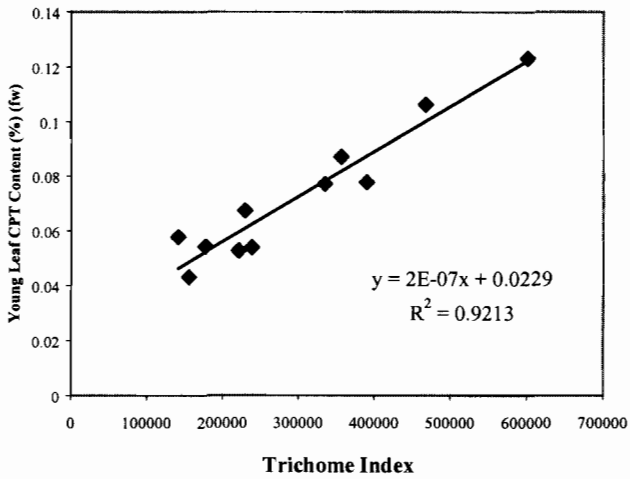


Figure 2. The linear relationship between trichome index and CPT content of young leaves of Camptotheca (Trichome Index = trichome volume × trichome density).

Understanding the defense mechanism is essential for developing strategies to enhance the yield of secondary metabolites. Existing studies usually focus on chemical analysis of a target tissue at a specific time, with no data available on time-course analysis of synchronical induction of chemicals in different tissues of plants. Thus, a general mechanism of induced-defense of plants remains elusive. The biosynthesis pathway of CPTs in Camptotheca is still not clear although it has been investigated for many years (Burnett et al., 1993; Lu and McKnight 1999). Based on synchronical time-course analysis of different parts of plants, Li and his colleagues recently found that chemical defense can be triggered by a change in plant auxin level through physical, biological, and ecological stresses in Camptotheca (Li et al., unpublished data). As the result of plant defense stimulation, CPTs can be significantly induced. The induced-defense seems only short-term but includes two steps: first an emergency response represented by increased alkaloid levels in the most protected young tissues due to local translocation from old tissues, and then a systemic response involving increased alkaloid levels in the whole plant. The mechanism found in Camptotheca may be conformed to a general model of induced-defense in plants.

In vitro Production of CPT

In vitro production of CPT by leaves, cotyledons, and other tissues has been studied in the United States, Japan, and Germany (Cooke 1973; Sakato

and Misawa 1974; Sakato et al., 1974; van Hengel et al., 1992; Wiedenfeld et al., 1997). Cooke (1973) found that CPT production in callus from both cambium and cotyledon was low for the first two weeks and absent after four weeks. Recently, Wiedenfield and his colleagues (1997) reported CPT and 10-hydroxycamptothecin present in shoot callus.

MATERIALS AND METHODS

Experimental Plants

The experimental Camptotheca plants for DNA and CPT analysis, including 25 populations, were grown in 2 gallon pots in a greenhouse in Nacogdoches, Texas, USA (Table 1). The seeds were sown in peat pots (Metro-Mix 366 growing medium) for germination in March 1995, 1996, and 1997, respectively. Seedlings were transferred to two gallon polyethylene pots filled with soil mix (hardwood bark:vermiculite = 3:1) after one-month's growth. The day/night temperature regime was maintained at 35.0/23.9°C (95/75°F) from March to November and 29.5/18.3°C (85/65°F) from December to February. Plants were watered once a day during the growing season and once every two days during the winter.

TABLE 1. Description of the experimental plant materials.

Accession Name	Origin	Collection Year	Seedling Number
C. acuminata var. acuminata			
HJ	Guangdong, China	1994	63
GN	Guangdong, China	1994	154
JT	Sichuan, China	1994	22
NJ	Jiangsu, China	1994	850
ZJ	Zhejiang, China	1994	220
AH	Anhui, China	1995	350
SH	Unknown, China	1994	20
CA	Shaanxi, China	1996	54
SA	San Antonio, TX, USA	1995	125
SM	Summerville, SC, USA	1994	50
HG	San Marino, CA, USA	1995	134
AT	Nacogdoches, TX, USA	1996	36
AB	Nacogdoches, TX, USA	1997	150
C. acuminata var. tenuifolia			
G9 *	Guangdong, China	1996	12
C. lowreyana var. lowreyana			
LY *	Guangdong, China	1994	22
D1 *	Guangdong, China	1994	35
D2 *	Guangdong, China	1994	10
G3 *	Guangdong, China	1996	26
G4 *	Guangdong, China	1996	50
C. lowreyana 'Ang'			
AG	Nacogdoches, TX, USA	1997	8
C. lowreyana 'Hicksii'			
HT	Nacogdoches, TX, USA	1998	3
C. lowreyana 'Katie'			
KT	Nacogdoches, TX, USA	1995	120
C. yunnanensis			
KM *	Yunnan, China	1996	11
XB	Yunnan, China	1996	54
YB	Yunnan, China	1994	20

Notes: * Natural origin, and others cultivated.

Genetic Diversity

The chloroplast DNA profiles were revealed by randomly amplified polymorphic DNA (RAPD) analysis (Williams et al., 1990). The RAPD analysis was conducted according to variation of RAPD markers within and among species/populations of Camptotheca with three replications for each sample. All the markers were scored by presence vs. absence of a specific amplification. The experiments were conducted at the SFA College of Forestry and documentation was performed at the SFA Science Research Center.

Sample Preparation

Experimental materials for DNA analyses were randomly collected from each of five plants for each population in Table 1. Three fully unfolded leaves were collected from each of five plants per population on the same day. The leaf materials collected from the same plant were ground with liquid nitrogen and stored in the freezer at -85°C.

DNA Extraction

Total DNA was isolated from young leaves using the CTAB (hexadecyltrimethylammonium bromide) procedure described by Doyle and Doyle (1990) with modification.

1. 0.5 g of powder per sample was quickly placed into 25 mL of preheated CTAB buffer (60°C) in a 50 mL falcon tube. The remaining leaf materials were returned to the freezer (-70°C) for use in the replication analysis.

2. Each sample was incubated in the CTAB buffer for 1-1.5 hr in a 60°C water bath with occasional mixing.
3. The samples were then stored at room temperature for about 10 min.
4. 25 mL of chloroform/Iso-amylalcohol (24:1 v/v) was added to each sample and mixed well, then evacuated.
5. The mixture was then centrifuged at 100 × g for about 10 min at room temperature.
6. The aqueous phase (upper phase) was removed with a 25 mL pipet and placed into a fresh tube.
7. 2/3 volume (14-16 mL) of cold isopropyl alcohol (-20°C) was added and mixed gently to precipitate the DNA in each tube.
8. DNA was recovered by one of two methods: (1) If the DNA was flocculate, the sample was centrifuged at 500 × g for 1-2 min or 1,600 × g for 10 min and the supernatant decanted; or (2) If the precipitate was not obvious, then the sample was centrifuged at 12,000 × g for 30 min and carefully decanted.
9. The supernatant was poured off and the tube was reversed on a paper towel to dry until the smell of isopropanol was gone.
10. 10-20 mL wash buffer was added to each tube, mixed gently and allowed to sit at room temperature for at least 20 min.
11. Each tube was centrifuged at 500 × g or 1,600 × g for 10 min at room temperature.

12. The supernatant was poured off carefully and the pellet was left in the tube to air dry briefly at room temperature.
13. The pellet was resuspended in 1 mL TE.
14. RNase A was added to a final concentration of 10-100 µg/mL (1-10 µl of 10 mg/mL stock) and the tube was incubated for 30 min at 37°C.
15. Each sample was diluted with 2X volume of TE (2 mL) and then 2.75X volume of 7.5 M ammonium acetate (pH 7.7) (2.3 mL) was added to a final concentration of 2.5 M, followed by the addition of 2.5X volume of cold ethanol (100%) (-20°C) (2.5 mL was added).
16. The DNA was centrifuged at 10,000 × g for 10 min or 1,600 × g for 30 min in a refrigerated centrifuge (4°C).
17. The air dried sample was resuspended in an appropriate amount of TE (~1 mL) and dispensed into 200 µl aliquots and stored at -20°C.
18. The concentration of template DNA was determined using a UV spectrometer at a wavelength of 260 nm.

Polymerase Chain Reaction Amplification

Twenty primers from two of each Operon kit A and kit B were used. Forty primers (Table 2), ten bases in length, were evaluated for suitability in a pilot survey in which three populations representing different species of Camptotheca were included. These primers were used for the polymerase chain reaction (PCR) based on the protocol of Williams et al., (1990) with optimization.

Amplification reactions were performed in a volume of 50 μl containing 10 mM Tris-Cl, pH 8.3, 50 mM KCl, 2.25 mM $MgCl_2$, 0.001% gelatin, 100 μM each of dATP, dCTP, dGTP, and dTTP, 12 picomoles (resuspended in 1 mL of water, using 0.5 μl per reaction) of a single 10-based primer, 50 ng of genomic DNA, and 1.0 units of Taq DNA polymerase (Promega Corp.). All reactions were overlaid with one drop of mineral oil before amplification in the thermocycler.

Amplification was performed in an Amplitron® II (Barnstead I Thermolyne, Dubuque, IA) programmed for preheat 3 min at 94°C, followed by 35 cycles of 1 min at 94°C, 1 min at 34°C, and 1 min and 30 sec at 72°C, followed by postdwell at 72 °C for 5 min. Amplification products were analyzed by electrophoresis in 1.5% agarose gel and detected by staining with ethidium bromide. A control lacking the DNA template was included in each amplification reaction. The products were viewed under ultraviolet light and photographed using Polaroid 665 film. Also Kodak' Digital Science™ 1D Image Analysis Software was used to store the images and to analyze the DNA electrophoresis gels for features including mass, molecular weight, intensity measurements and mobility values.

Nomenclature

Each amplified band was named by the primer used and its size in bp. For example, OPA02-2500 refers to the 2,500 bp band amplified by primer OPA02.

TABLE 2. Random oligonucleotide primer sequences of Operon Kit A and Kit B.

Kit A		Kit B	
Code	**5' to 3'**	**Code**	**5' to 3'**
OPA-01	CAGGCCCTTC	OPB-01	GTTTCGCTCC
OPA-02	TGCCGAGCTG	OPB-02	TGATCCCTGG
OPA-03	AGTCAGCCAC	OPB-03	CATCCCCCTG
OPA-04	AATCGGGCTG	OPB-04	GGACTGGAGT
OPA-05	AGGGGTCTTG	OPB-05	TGCGCCCTTC
OPA-06	GGTCCCTGAC	OPB-06	TGCTCTGCCC
OPA-07	GAAACGGGTG	OPB-07	GGTGACGCAG
OPA-08	GTGACGTAGG	OPB-08	GTCCACACGG
OPA-09	GGGTAACGCC	OPB-09	TGGGGGACTC
OPA-10	GTGATCGCAG	OPB-10	CTGCTGGGAC
OPA-11	CAATCGCCGT	OPB-11	GTAGACCCGT
OPA-12	TCGGCGATAG	OPB-12	CCTTGACGCA
OPA-13	CAGCACCCAC	OPB-13	TTCCCCCGCT
OPA-14	TCTGTGCTGG	OPB-14	TCCGCTCTGG
OPA-15	TTCCGAACCC	OPB-15	GGAGGGTGTT
OPA-16	AGCCAGCGAA	OPB-16	TTTGCCCGGA
OPA-17	GACCGCTTGT	OPB-17	AGGGAACGAG
OPA-18	AGGTGACCGT	OPB-18	CCACAGCAGT
OPA-19	CAAACGTCGG	OPB-19	ACCCCCGAAG
OPA-20	GTTGCGATCC	OPB-20	GGACCCTTAC

Data Analysis

Photographs from ethidium bromide stained agarose gels were used to score the data for RAPD analysis. Starting from the higher molecular weight product to lower molecular weight product, the amplified fragments were designated as described in the nomenclature section. The presence of a product was identified as 1 and its absence was recognized as 0. In this way, data were scored for all genotypes, their amplification products, and primers. The genetic data obtained was summarized and evaluated using the software package POPGENE (Yeh et al.,1997).

Within populations, two common estimates of genetic variability were computed for each population and then averaged for cultivars, varieties, and species. These estimates included percentage of polymorphic loci (*P*), which is defined as

$$P = \text{number of polymorphic loci/total number of loci,}$$

and Shannon's (1949) information index (*I*) for each locus *I*, which is defined as

$$I_i = -\Sigma\pi\log_2\pi,$$

where π is the phenotypic frequencies, and averaged across loci:

$$I = (1/L)\times\Sigma I_i,$$

where L is the number of loci.

Total gene diversity (H_T), gene diversity within populations (H_S), gene diversity among populations (D_{ST}) and the proportion of diversity among populations (G_{ST}, where $G_{ST} = D_{ST}/H_T$) were calculated according to Nei (1973, 1977). These statistics were also averaged across all polymorphic loci to obtain species-level and genus-level estimates of genetic diversity.

The number of migrants per generation (Nm) was calculated as the estimate of gene flow from G_{ST}, $Nm = 0.5(1- G_{ST})/ G_{ST}$. In other words, the movement of one individual per generation between populations is sufficient to prevent substantial differentiation between those populations. This result, independent of migrants in a population (denoted by m), is counteracted by the

force of genetic drift, which is proportional to the inverse of the population size, denoted by N (McDonald 1993).

Nei's genetic identity (S) and genetic distance (D) were calculated between 25 populations (Nei 1972). Nei's genetic distance between populations X and Y is defined as

$$D = -\ln(J_{XY}/\sqrt{J_X J_Y}),$$

where J_X, J_Y, and J_{XY} are the means of Σx_i^2, Σy_i^2, and $\Sigma x_i y_i$ over all loci studied, respectively. x_i and y_i are the frequencies of the ith allele in populations X and Y, respectively. Genetic identity is defined as

$$S = J_{XY}/\sqrt{J_X J_Y}$$

A dendrogram based on Nei's genetic distance was constructed using the unweighted pair-group arithmetic average (UPGMA) clustering (Sneath and Sokal 1973).

Camptothecin Variation

Sample Preparation

A. Within and among populations

In May 2000, young leaves were collected from the top three stems of each of three randomly selected two-year-old plants. The experimental materials included 11 populations of different species, varieties, and cultivars: C. acuminata (NJ, AH, AT, and AB), C. acuminata var. tenuifolia (G9), C. lowreyana

(LY), C. lowreyana 'Katie' (KT), C. lowreyana 'Hicksii' (HT), and C. yunnanensis (YB, KM, and XB) (see Table 1).

B. Within plant

Experimental materials were randomly collected from five plants of C. acuminata (seed source: SFA 94-03). Leaf, stem, and root materials were collected in May, wood and bark samples were collected in August, and fruit samples were collected in June, August, and October, respectively, of 2000.

C. With season

The experimental plants of C. acuminata were germinated from seeds collected from Anhui, China in 1995. In 2000, the intact young tissues were collected monthly (from March to October) from each of the same five four-year old plants at the SFA campus. Three flower/fruit inflorescences were collected from the same tree at the following ages: week 1, 2, 8, 10, 13, and 16. All fruits (30-40) in the same inflorescence were prepared as one sample for CPT analysis.

D. With age

The seed source for the experimental plants of C. acuminata is a single tree in San Antonio, Texas. In March 1993, several seedlings propagated by cuttings were planted on the SFA campus. From 1996 to 1999, seeds were collected from these trees each fall and sowed in the field in Nacogdoches the next spring. In May 1999, five plants per age class were randomly selected from

one, two, and three year-old seedlings, respectively, and two seven year-old parent trees. Each sample included five young leaves collected from the top five branches of the plants. A second set of samples was also collected from the same plants in July 2000.

E. With stress (T-pruning treatment)

The plants to be exposed to different pruning treatments were also grown in the greenhouse under similar conditions for one year and then were transferred outdoors. The AH seeds of C. acuminata were sown on March 11, 1996. In March 11, 1997, 207 plants were transferred into two-gallon pots in the field in Nacogdoches. Plants were watered once a day during growing season and once every two days in winter. These plants were randomly assigned to three groups containing 69 plants each: control, T-pruning treatment I (at 30 cm height), and T-pruning treatment II (at 40 cm height).

F. Determination of preservation methods

The following experiments were conducted to determine the best method of preservation for CPT from plant materials. On May 31, 2000, 15 intact young tissues (clippings) were collected from each of six plants of C. acuminata with the same seed source (AH). They were weighed and randomly classified into five groups with three clippings each. The first three clippings were immediately extracted and analyzed. The second, third, fourth and fifth groups of three clippings were frozen immediately in the freezer (-85°C) for 48 hrs, vacuum-dried

for 48 hrs, dried in an oven (65°C) for 48 hrs, or dried by air under sun for 72 hrs, respectively. Then from each sample, plant material equivalent to 4 g of fresh weight were used for separate CPT analysis. The other five plants served as five replications. The results showed that fresh, frozen, and vacuum-dry materials have no significant difference in CPT yield between each other and provide the best preservation of CPT content (Table 3). Air-dried and oven-dried materials lost about 30% of their CPT content, compared with fresh or frozen materials. Clearly, freeze-treatment not only resulted in the best CPT preservation but also provided for long-term sample storage. Therefore, freeze-preservation was used for all further CPT analysis in the present study.

TABLE 3. CPT contents of intact young tissues of C. acuminata preserved by different methods (6 replications, fresh weight)

Preservation Method	CPT Content (% ± s.d.)
Fresh	0.0339 ± 0.0039^{a}
Freeze	0.0336 ± 0.0035^{a}
Vacuum-dry	0.0328 ± 0.0022^{a}
Air-dry	0.0233 ± 0.0045^{b}
Oven-dry (65°C)	0.0228 ± 0.0059^{b}

The CPT content with the same letter are not significantly different.

Determination of Camptothecin Content

The collected plant samples were separately weighed and ground by using liquid nitrogen and then stored in freezer (-85 °C). CPT content was expressed as the percentage of fresh weight of the plant material. Each sample was extracted three times. An ASE 200 Accelerated Solvent Extractor (Dionex Corp., Sunnyvale, CA) was used for the CPT extraction. One or four grams of

material from each sample was loaded in the 22 mL cell. Disposable cellulose filters (Dionex) were inserted in the bottom of the sample cell before filling and in the top of the cell after filling to prevent blockage of the bottom cap's stainless steel frit. Sand was used to fill the void between the top filter and the top opening of the cell to reduce the amount of solvent used during the extraction. A third filter was placed on top of the sand before screwing and hand tightening the top cap onto the cell body. The filled cells were loaded into the tray slots in numerical order. Then 60 mL clear vials were used to collect the extract. Ninety-five percent EtOH was used as the solvent. The extraction parameters were as follows: temperature 85 °C, pressure 1500 psi, static time 30 min, flush 100% volume, purge 120 s, and cycle 1. The ethanol extract was adjusted to 40 mL with acetonitrile, then 2 mL of the extraction was added to a microcentrifuge tube, which was diluted to 5 mL with acetonitrile and centrifuged at 5,000 rpm for 4 minutes. The upper liquid fraction was analyzed by HPLC (high performance liquid chromatography).

Reverse phase HPLC (HP 1100) analysis of samples was carried out under the following conditions: column temperature 40 °C, flow rate 1 mL/min, and CH_3OH-H_2O-CH_3CN (15:75:10---25:45:35) as the gradient mobile system within 15 min. Ten microliters of the solution was injected into the column (C-18, 5μ, 250 × 4.6 mm) and equilibrated with 77% water (Nanapure) with 13% acetonitrile (HPLC grade) and 10% methanol (HPLC grade) as the mobile phase for an initial period

of 5 minutes, and then the mobile phase was increased to 35% water, 35% acetonitrile and 30% methanol. A complete HPLC spectrum was obtained in 15 minutes. The CPT peak (R_t at 7 min), detected at 254 nm (DAD detector), of the sample was identified by comparison with that of the standard (Sigma). The integrating software used was EZChrome (Shimadzu, Japan).

Data Analysis

CPT data from the different populations/treatments were analyzed by one-way ANOVA at 0.05 significant level using the SAS system (version 8, 1999).

RESULTS AND DISCUSSION

Genetic Diversity

Detailed genetic databases are important in the management of endangered species. The level of genetic variation may influence a population's growth rate and ability to adapt to changing environmental conditions (Delany et al., 2000).

Primer Selection

Initially the level of polymorphism detected with RAPD markers was assayed in three populations representing different species of Camptotheca. Of the 40 primers screened in this study, three (OPA02, OPA03, and OPA04) were selected because they all revealed multibanded fingerprints, which were clearly scorable. Following primer selection, the above method with selected primers was used in the DNA amplification of all populations and treatments. The number of polymorphic bands for each primer varied from 12 (OPA03) to 16 (OPA02 and OPA04) bands, with an average of 15 bands per primer. The size of the amplified fragments ranged from 268bp to 4,411bp (Table 4).

TABLE 4. List of selected primers and their sequences that produced polymorphic markers among the Camptotheca populations studied.

Primers	Number of polymorphic fragments	Size range of the polymorphic scored fragments (bp)
OPA-02	16	271-3015
OPA-03	12	270-2423
OPA-04	16	268-4411
Total/Range	44	268-4411

Genetic Polymorphism

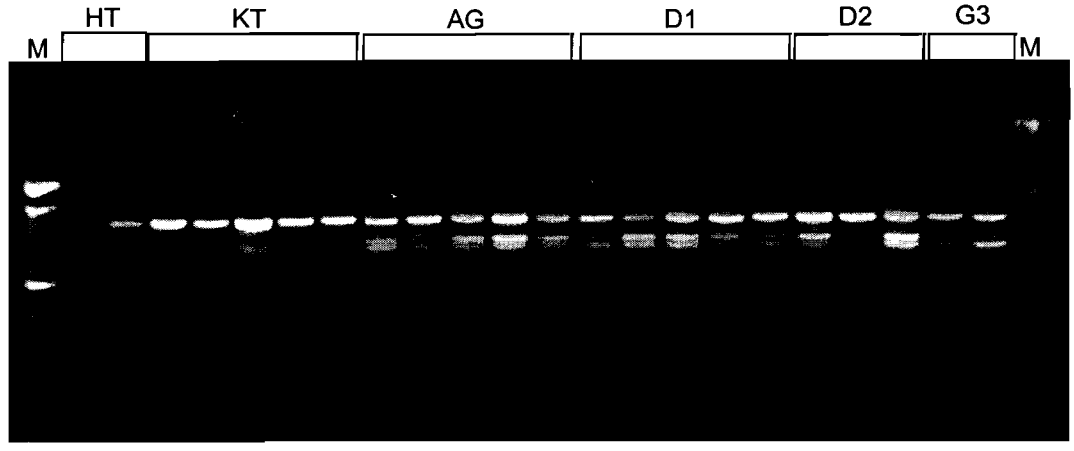
The RAPD amplification products generated can be classified into two types: constant (monomorphic) and variable (polymorphic) (Orozco-Castillo et al, 1994). This difference can be used to examine and establish systematic relationships (Hadrys et al., 1992). Bands were defined as polymorphic if the mean fragment frequency was not fixed (i.e., 1 or 0) (Boehm et al, 1999).

The optimized PCR protocol resulted in highly reproducible banding patterns. A total of 46 strongly amplified and highly reproducible scorable bands were generated using these three selected primers (Appendix A). Among these 46 bands, 44 (95.7%) were polymorphic and two were monomorphic among all the populations tested (Appendix A) according to the allelic frequencies. Allelic frequencies of the 44 polymorphic loci are summarized for each variety and species in Appendix B. Allele frequencies for each population can be obtained from the author. Most of the populations studied possessed unique combinations of bands, thereby permitting their identification. It was noteworthy that the first

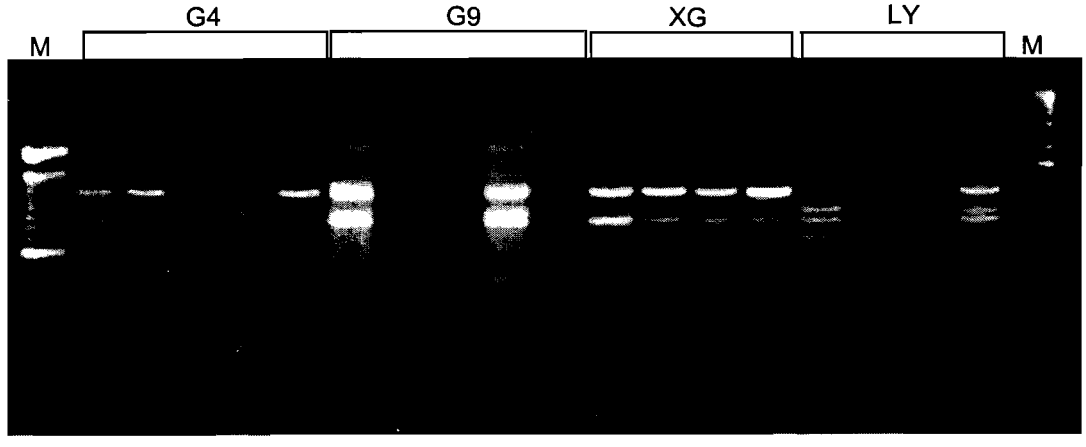
band of primer OPA03 (OPA03-2423) was present in all individuals of the cultivar 'Katie' (KT) but completely absent in the other populations. The 16th band of primer OPA04 (OPA04-268) was present exclusively in all individuals of the HJ population of C. acuminata. The fifth band of primer OPA03 (OPA03-1460) was absent in all individuals of the cultivar 'Ang' (AG) but present in other Camptotheca populations. These distinctive bands can be used to identify these three populations, respectively.

RAPD profiles resulted from the use primers OPA02, OPA03, and OPA04 (Figure 3-5). The polymorphism revealed in Camptotheca populations by amplification of arbitrary primers is extensive. By using the three primers to analyze diversity within each of 13 populations of C. acuminata, nine populations of C. lowreyana, and three populations of C. yunnanensis populations, average estimates of genetic diversity were obtained (Table 5), which provide useful information on population structure at most collection sites.

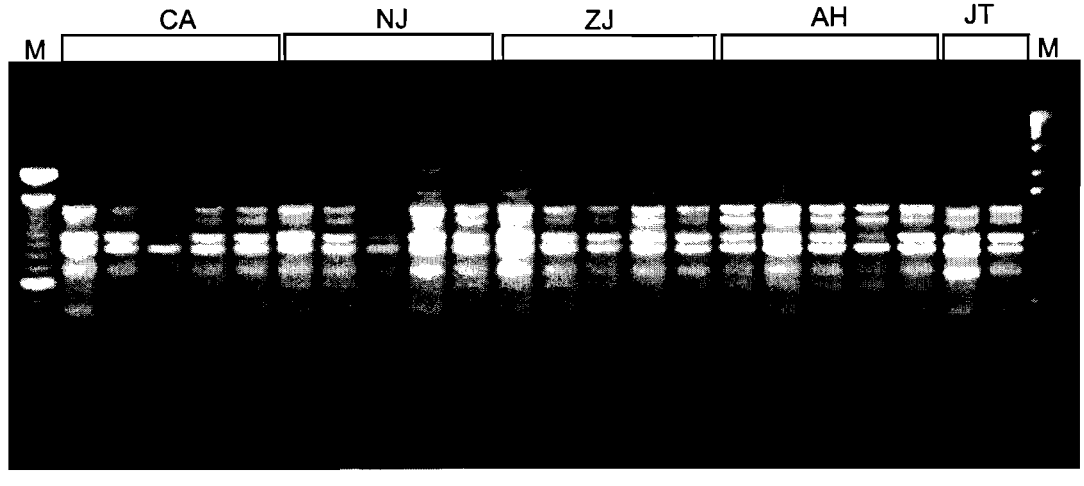
a



b



c



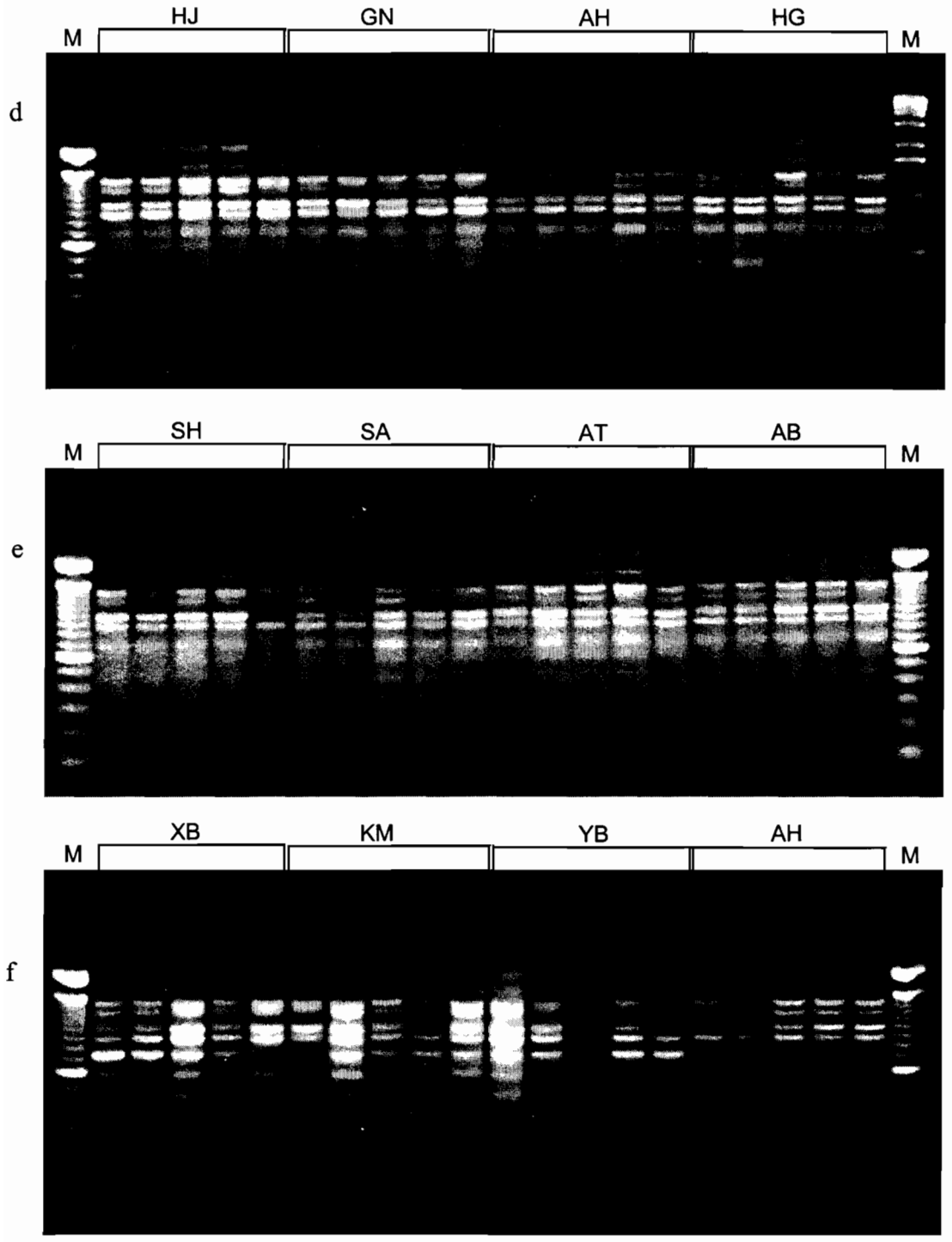
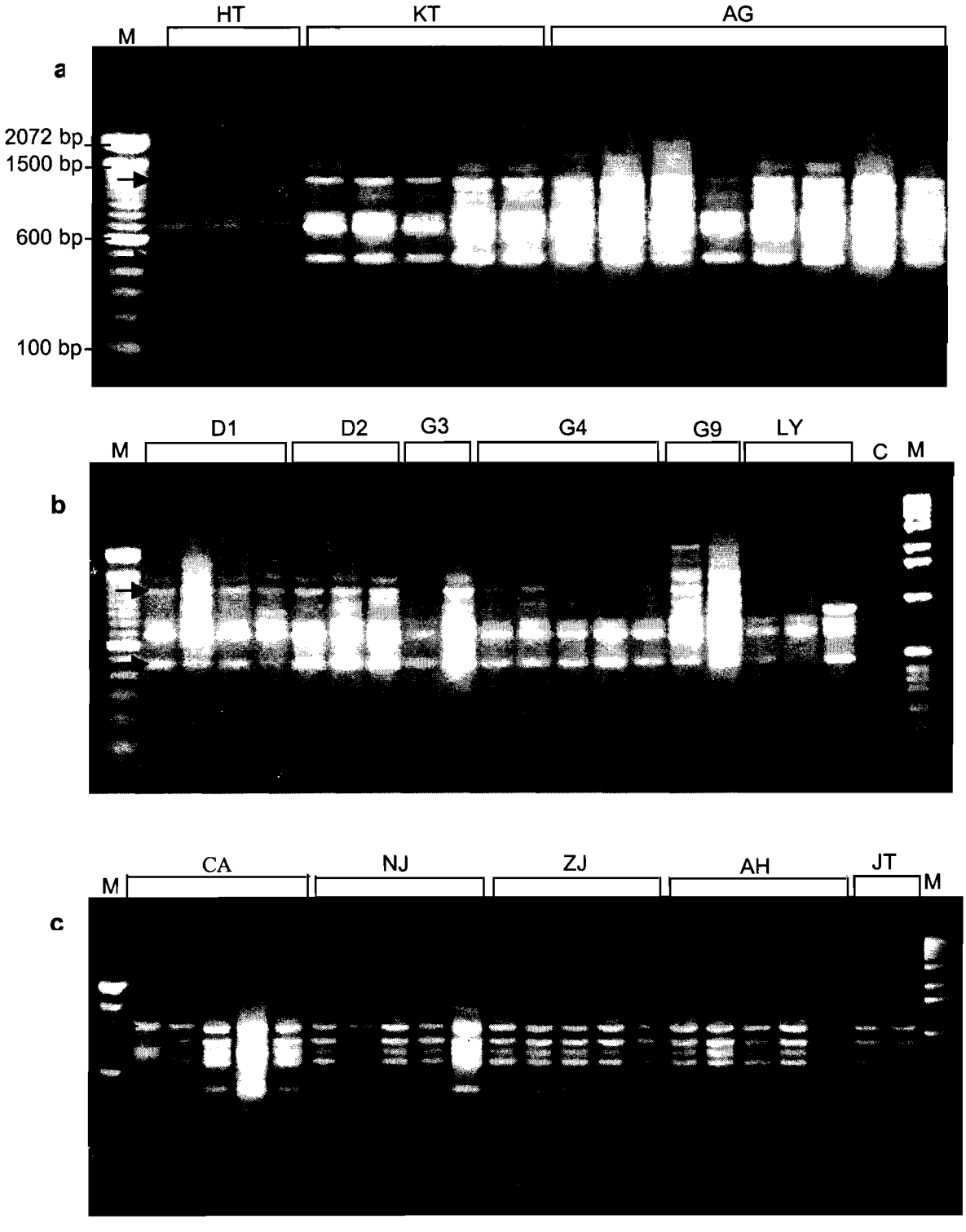


Figure 3. RAPD profile of 25 populations of Camptotheca using primer OPA02 (a-f). Lane M on the left is a 100 bp ladder marker and on the right is a 1Kb or 100bp ladder marker.


a
M
HT
KT
AG
2072 bp
1500 bp
600 bp
100 bp
b
M
D1
D2
G3
G4
G9
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C
M
c
M
CA
NJ
ZJ
AH
JT
M

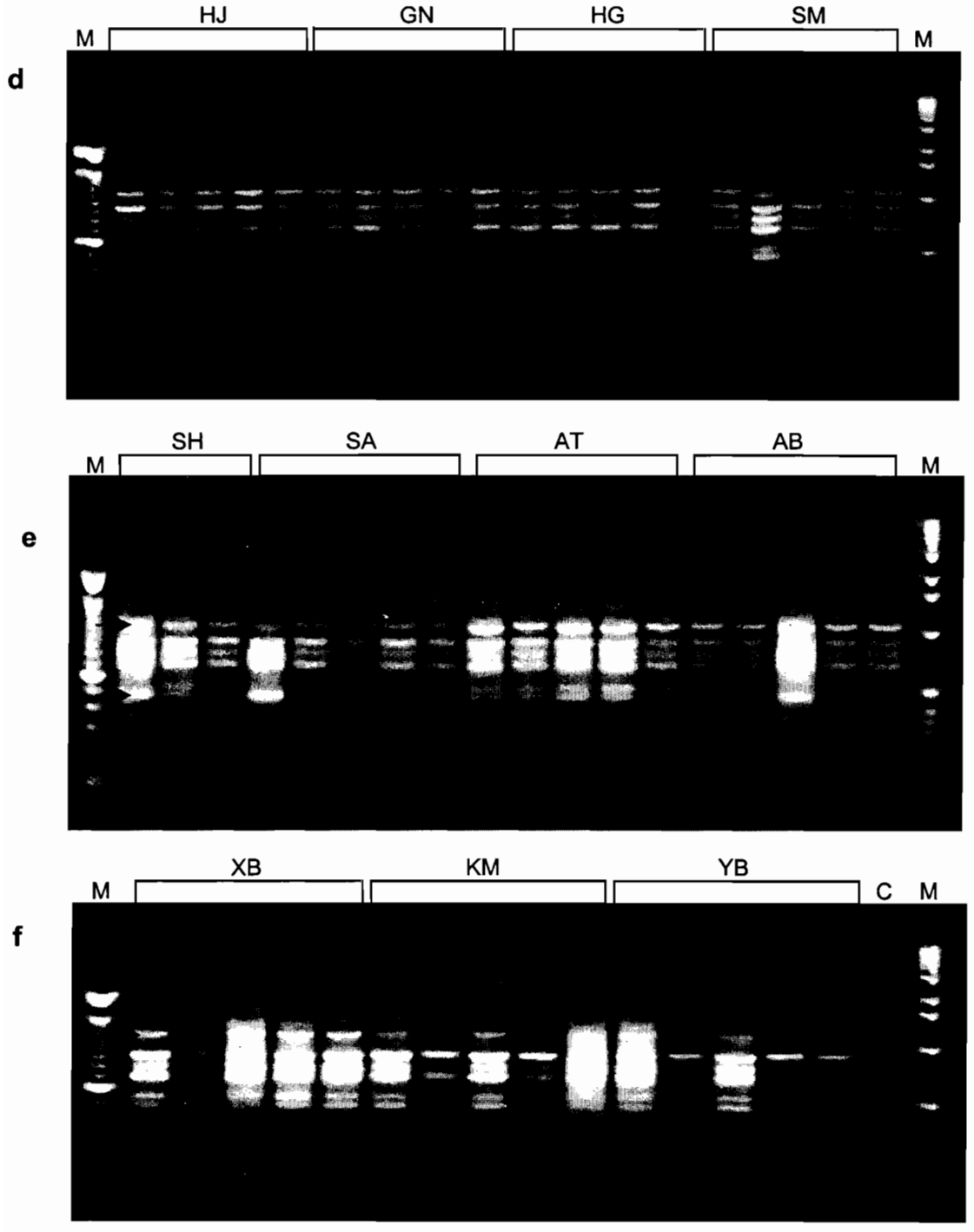
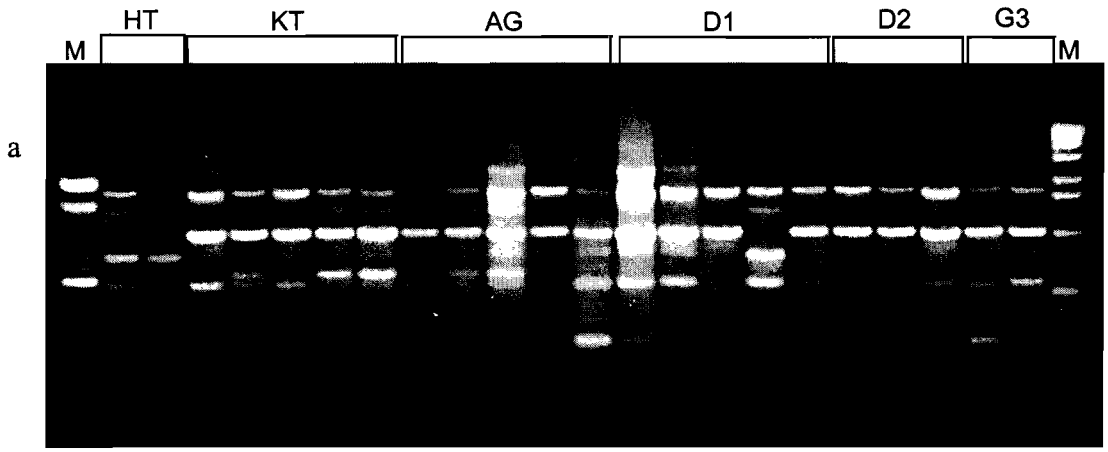
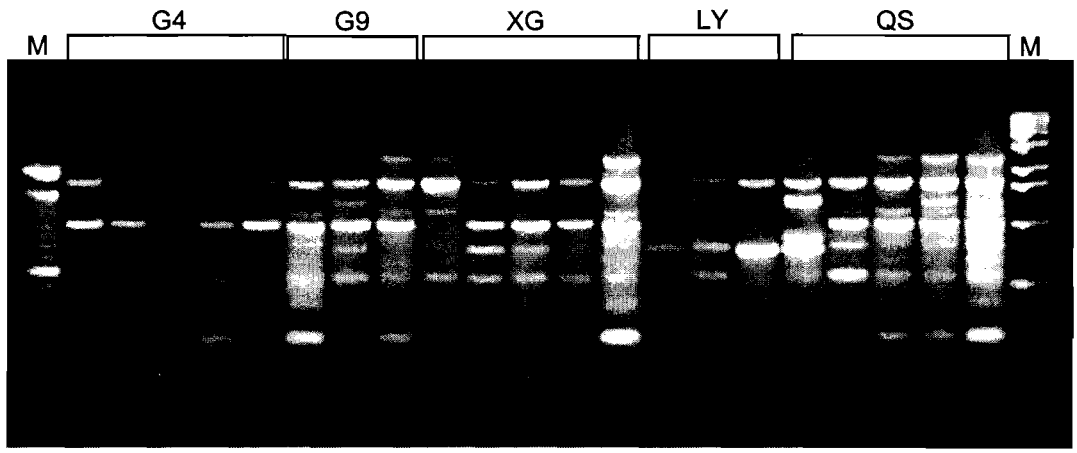


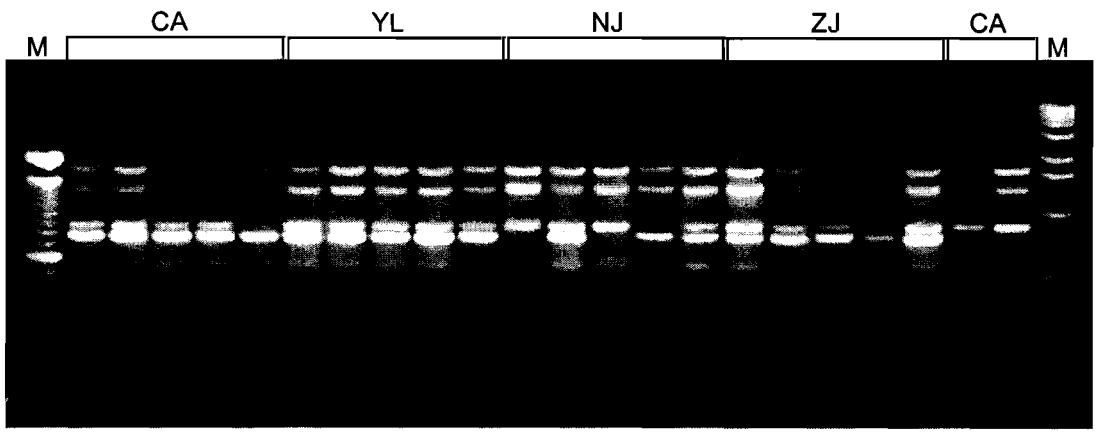
Figure 4. RAPD profile of 25 populations of Camptotheca using primer OPA03 (a-f). Lane M on the left is a 100 bp ladder marker and on the right is a 1Kb ladder marker. Lane C is a negative control lane without any genomic DNA. The two fragments, OPA03-1100 and OPA03-480, were found in common within all populations (arrows).



a



b



c

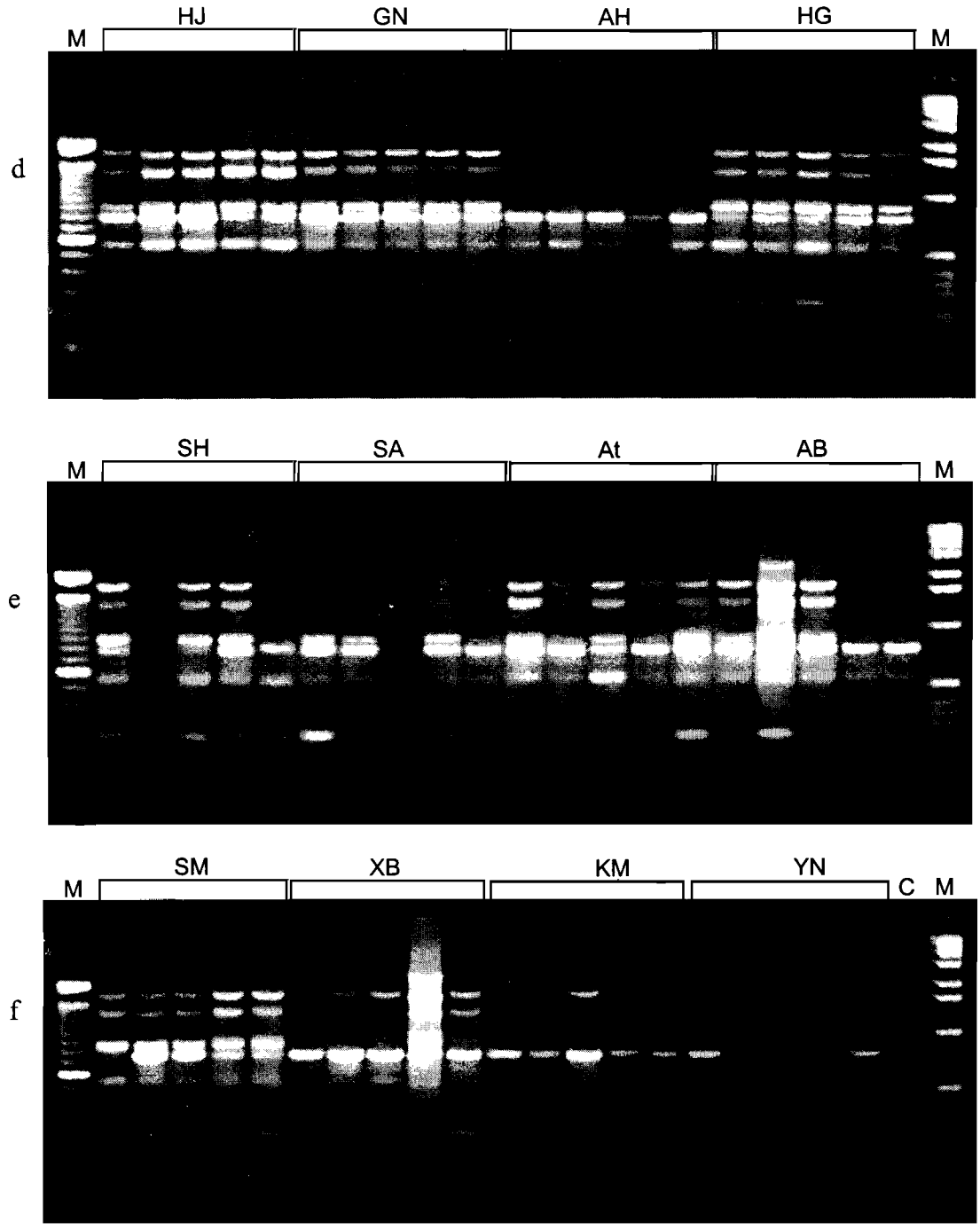


Figure 5. RAPD profile of 25 populations of Camptotheca using primer OPA04 (a-f). Lane M on the left is a 100 bp ladder marker and on the right is a 1Kb ladder marker. Lane C is a negative control lane without any genomic DNA.

Data on the number and proportion of polymorphic RAPD loci detected with each primer in the C. acuminata, C. lowreyana, and C. yunnanensis populations are shown in Table 5, with OPA02 and OPA04 detecting the greatest number of scorable polymorphic loci.

The number and proportion of polymorphic loci for each population are also shown in Table 5. KT and G3 populations exhibited the highest level of variability. In contrast, the G9 population exhibited the lowest level of variability with no primer detecting any polymorphic amplification product.

Plant populations under different environmental selection pressures generally show phenotypic differences. Such phenotypic differences may be the result of phenotypic plasticity and/or genetic diversification existing among populations (Wen and Hsiao 1999).

TABLE 5. Polymorphic loci detected with three primers for 13 populations of C. acuminata, nine of C. lowreyana, and three of C. yunnanensis and the total number of polymophic loci scored in all the populations (proportion of polymorphic loci).

Cultivars	Primer			Total number polymorphic loci
	OPA02	OPA03	OPA04	
HT	1 (0.063)	0 (0.000)	0 (0.000)	1 (0.022)
KT	5 (0.313)	0 (0.000)	3 (0.177)	8 (0.174)
AG	4 (0.250)	1 (0.071)	2 (0.118)	6 (0.130)
D1	1 (0.063)	2 (0.143)	4 (0.235)	3 (0.065)
D2	3 (0.188)	0 (0.000)	0 (0.000)	3 (0.065)
G3	2 (0.125)	2 (0.143)	4 (0.235)	8 (0.174)
G4	5 (0.313)	1 (0.071)	0 (0.000)	6 (0.130)
G9	0 (0.000)	0 (0.000)	0 (0.000)	0 (0.000)
LY	4 (0.250)	0 (0.000)	0 (0.000)	4 (0.087)
CA	0 (0.000)	5 (0.357)	0 (0.000)	5 (0.109)
NJ	0 (0.000)	3 (0.214)	3 (0.177)	6 (0.130)
ZJ	0 (0.000)	2 (0.143)	2 (0.118)	4 (0.087)
AH	0 (0.000)	1 (0.071)	0 (0.000)	1 (0.022)
JT	0 (0.000)	0 (0.000)	1 (0.059)	1 (0.022)
HJ	1 (0.063)	0 (0.000)	1 (0.059)	2 (0.043)
GN	1 (0.063)	0 (0.000)	0 (0.000)	1 (0.022)
HG	2 (0.125)	0 (0.000)	0 (0.000)	2 (0.043)
SM	1 (0.063)	0 (0.000)	3 (0.177)	4 (0.087)
SH	0 (0.000)	3 (0.214)	1 (0.059)	3 (0.065)
SA	1 (0.063)	2 (0.143)	1 (0.059)	2 (0.043)
AT	1 (0.063)	0 (0.000)	1 (0.059)	1 (0.022)
AB	1 (0.063)	3 (0.214)	6 (0.353)	1 (0.022)
XB	1 (0.063)	0 (0.000)	3 (0.177)	4 (0.087)
KM	1 (0.063)	0 (0.000)	5 (0.294)	6 (0.130)
YB	5 (0.313)	1 (0.071)	0 (0.000)	6 (0.130)
Total	16 (1.000)	12 (0.857)	16 (1.000)	44 (0.957)

Genetic diversity

A. Genus

To assess the overall distribution of variability between and within all populations gene diversity statistics are calculated (Nei 1973) for each RAPD locus. Gene diversity statistics were presented in Appendix C1. The partitioning of gene diversity within and among populations is reflected in G_{ST} values (Hamrick and Godt 1989). The total observed diversity estimates (H_T) were partitioned into within population diversity (H_S) and between population diversity component (D_{ST}), where $H_T = H_S + D_{ST}$. Gene diversity between populations was expressed relative to total population diversity as $G_{ST} = D_{ST}/H_T$.

The distribution of variability differed among polymorphic loci. Total gene diversity (H_T) ranged from the lowest value in locus OPA04-4411 (0.0084), detecting the least variability, to the highest values in locus OPA04-880 (0.4982), detecting the most variability. Examination of the gene diversity statistics for each locus indicates an extremely high G_{ST} (1.0000) at OPA02-2400, OPA03-2423, OPA03-910, and OPA04-268 (Appendix C1). There was little heterogeneity among loci in patterns of diversity; almost all were characterized by high G_{ST} values, suggesting that genetic patterns across loci result from general influences in the entire genome.

An examination of the proportion of diversity present within populations (1-G_{ST}) compared to between populations (G_{ST}) indicated that, on average, more diversity was detected between populations (84.9%) (Table 6). Most gene

diversity is partitioned among rather than within populations, regardless of the differences in absolute levels of diversity (Table 6).

TABLE 6. Gene diversity statistics for 25 Camptotheca populations examined with 44 RAPD polymorphic loci detected with three primers

Species	H_T	H_S	D_{ST}	G_{ST}	Nm
C. acuminata	0.1176	0.0249	0.0927	0.7881	0.1344
C. lowreyana	0.1914	0.0429	0.1485	0.7760	0.1444
C. yunnanensis	0.0647	0.0354	0.0293	0.4529	0.6040
Total	0.2113	0.0319	0.1794	0.8490	0.0889

Notes: H_T is total variation in all populations, H_S is the average gene diversity found within populations, D_{ST} is the average gene diversity among populations, G_{ST}, equivalent to D_{ST}/H_T, is the proportion of total gene diversity due to differences among populations, and Nm is the estimate of gene flow from Gst, Nm = 0.5(1-Gst)/Gst.

Gene diversity statistics corroborate the pattern of low within-population levels of variation despite high levels of total polymorphism (Table 6). Total gene diversity was high, within-population diversity was low, and most of the genetic diversity occurred between populations. The value of G_{ST} obtained for 25 Camptotheca populations was relatively high in relation to other studies (Rowden 1999; Elisens et al., 1992) using RAPDs. This suggests that at some stage, isolation events have prevented gene flow (Nm = 0.0889) and subsequently, genetic drift has led to considerable population differentiation in Camptotheca.

B. Species

Two common estimates of genetic variability for populations, varieties, and species are listed in Table 7. C. lowreyana was found to have the highest within-species variability (P=63.64 and I=0.2980), following by C. acuminata (P=63.64 and I=0.1753), with C. yunnanensis, the lowest (P=27.27 and I=0.1031). This result is consistent with phenotypic variation analysis (Li et al., 2000).

For C. lowreyana, the highest genetic diversity is found in its natural variety var. lowreyana (P=59.09 and I=0.2468) (Table 7). This variety also shows greater phenotypic variations among populations (Li et al., 2000). The experimental samples of var. lowreyana represent the different natural populations from Guangdong Province, China. The genetic diversity is much smaller within each cultivar (cultivars 'Hicksii', 'Katie', and 'Ang') because the plants of each cultivar were asexually propagated from their single "parent" plants. The cultivar 'Hicksii' exhibited the lowest diversity (P=2.27 and I=0.0137). C. lowreyana has not only the highest H_T value (H_T = 0.1914) but also a greater proportion of gene diversity distributed among populations (G_{ST} = 0.7760) (Table 6).

TABLE 7. Proportion of polymorphic loci (P) and shannon's information index (I) for Camptotheca as a whole, each species, variety and cultivar of C. acuminata and C. lowreyana, and populations within each variety.

Species	P	I
C. acuminata	63.64	0.1753
var. acuminata	47.73	0.1392
CA	11.36	0.0580
NJ	13.64	0.0774
ZJ	9.09	0.0454
AH	2.27	0.0158
JT	2.27	0.0912
HJ	4.55	0.0271
GN	2.27	0.0121
HG	4.55	0.0243
SM	9.09	0.0520
SH	6.82	0.1412
SA	4.55	0.0315
AT	2.27	0.0156
AB	22.73	0.1102
var. tenuifolia (G9)	0	---
C. lowreyana	63.64	0.2980
Var. lowreyana	59.09	0.2468
D1	6.82	0.0371
D2	6.82	0.0418
G3	18.18	0.1099
G4	13.64	0.0789
LY	9.09	0.0550
'Ang' (AG)	13.64	0.0743
'Hicksii' (HT)	2.27	0.0137
'Katie' (KT)	18.18	0.0948
C. yunnanensis	27.27	0.1031
XB	9.09	0.0406
KM	13.64	0.0549
YB	13.64	0.0721
Total	100.00	0.3228

Within C. acuminata, var. acuminata exhibited the most variation (P=47.73 and I=0.1392) (Table 7). Because the other variety, var. tenuifolia, has only one population available for analysis, it is not known how representative of the variant it is. The samples of var. acuminata represent only cultivated plants because no wild population has been identified in its native China to date (Li et al., 2000). Some of these introductions can be traced back to the seed source in China. Therefore, it is reasonable the species has low genetic diversity. For var. acuminata, population AB (P=22.73 and I=0.1102) has discernibly more genetic variation. In contrast, the other 12 populations display lower variations (P=2.27-11.36). AH, JT, GN, and AT have the lowest variations within C. acuminata because the seeds of each population originated from a single parent tree. Although it has the lowest genetic diversity, C. acuminata has the greatest proportion of gene diversity distributed among populations (G_{ST} = 0.7881) (Table 6).

For C. yunnanensis, the difference among three populations was relatively small. The tested materials of C. yunnanensis represent the only three known populations in China (Li et al., 2000). Camptotheca yunnanensis not only had the lowest H_T value (0.0647) but also the lowest proportion of gene diversity distributed among populations (G_{ST} = 0.4523) as compared with C. acuminata and C. lowreyana.

Genetic diversity for all polymorphic loci in each species was estimated (Appendix C2). Among polymorphic loci, total genetic diversity (H_T) for C. lowreyana ranged from 0.0518 (OPA04-1650) to the highest values in OPA02-2400 (0.4938) and OPA02-1500 (0.4999).

The G_{ST} values of C. acuminata and C. lowreyana were relatively higher than the mean value for selfing species. Even the Gst value of C. yunnanensis (G_{ST} = 0.4523) approached the mean value (G_{ST} = 0.51) estimated for selfing species (Hamrick and Godt 1989). The higher G_{ST} values indicated that, of the total genetic variation, the differences among populations are very significant in each species. The calculated Nm values, ranging from 0.1344 for C. acuminata to 0.6040 for C. yunnanensis, were all less than 1.0, which is commonly taken as the breakpoint below which genetic drift can play a major role in determining the distribution of genetic variation among populational subdivisions (Wright 1951).

In this study, the level and structure of genetic variation of C. acuminata and C. lowreyana was described from analysis with RAPD markers. Within-population gene diversity was found to be relatively low compared with other tree species. Population differentiation was found to be higher than other species with similar breeding system. It appeared that fragmentation has caused gene flow to become low enough for factors such as genetic drift and possible inbreeding depression to cause this differentiation. All populations were therefore distinctive genetically and each should be considered as a management unit.

C. Genetic identity and distance

Nei's genetic identity (I) and distance (D) coefficients for all 25 populations of Camptotheca are shown in Appendix D. The mean values for Nei's genetic identity and distance for pairwise combinations of species and populations in Camptotheca are summarized in Table 8. The genetic distance scale runs from 0 (identical) to 1 (different for all criteria studied) and a figure of 0.2824 or more differentiates different species. Exceptions exist between C. yunnanensis and C. acuminata (D = 0.1916), and also between C. yunnanensis and C. acuminata var. acuminata (D = 0.1767). Interspecific comparisons indicated that C. yunnanensis was most similar genetically to C. acuminata (D = 0.1916). The highest interspecific identities were between C. yunanensis and C. acuminata var. acuminata (D = 0.1767). The greatest interspecific distances were between C. yunanensis and C. lowreyana 'Ang' (D = 0.4625) and between C. acuminata var. tenuifolia and C. lowreyana 'Katie' (D = 0.4614).

Distance values of 0.1973 or less were obtained within each species and variety, the smallest value arising within C. yunnanensis (D = 0.0420), indicating that populations of C. yunnanensis were the most similar genetically. The genetic distance value obtained between varieties of C. acuminata var. acuminata and var. tenuifolia was higher (D = 0.4340) than most species distances. The genetic distance values obtained between varieties and cultivars of C. lowreyana ranged from 0.1684 to 0.2509. Camptotheca lowreyana var. lowreyana was closer to

cultivar 'Hicksii' (D = 0.1684) than to cultivars 'Katie' and 'Ang'. In contrast, the greatest distance existed between cultivars 'Katie' and 'Ang' (D = 0.2509).

The ranges of values obtained between species would suggest that, of all the species studied, C. yunnanensis was the closest relative to C. acuminata var. acuminata.

Table 8. Mean values for Nei's genetic distance for pairwise combinations of species, varieties, and cultivars in Camptotheca.

Species/Variety	C. acuminata	C. acuminata var. acuminata	C. acuminata var. tenuifolia	C. lowreyana	C. lowreyana var. lowreyana	C. lowreyana 'Hicksii'	C. lowreyana 'Katie'	C. lowreyana 'Ang'	C. yunnanensis
C. acuminata	0.1078	———	———	0.3162	0.3004	0.2431	0.3861	0.3978	0.1916
C. acuminata var. acuminata		0.0623	0.4340	0.3103	0.2940	0.2309	0.3803	0.4006	0.1767
C. acuminata var. tenuifolia			———	0.3932	0.3842	0.4021	0.4614	0.3611	0.3862
C. lowreyana				0.1973	———	———	———	———	0.3354
C. lowreyana var. lowreyana					0.1714	0.1684	0.2139	0.2353	0.3157
C. lowreyana 'Hicksii'						———	0.2323	0.2386	0.2824
C. lowreyana 'Katie'							———	0.2509	0.3595
C. lowreyana 'Ang'								———	0.4625
C. yunnanensis									0.0420

Note: Figure underlined is the genetic distance among populations within the taxon.

D. Cluster analysis

Cluster analysis is a standard method for analyzing the relatedness of individuals (and hence grouping them) from measured data. Cluster analysis has the advantage over some other grouping methods, for example principal component analysis, in that the number of related groups under study does not have to be known, or suspected, in order to carry out the analysis. The main assumption made is that two individuals, or cultivars, which group together at a particular level, share a common ancestor more recently than those that join at a higher level.

Cluster analysis of the genetic distance values was conducted to generate a dendrogram indicating relationships between the Camptotheca populations studied (see Figure 6). The dendrogram generated was in general agreement with Li's taxonomic treatment of Camptotheca (Li 1997, 2001; Li et al., 2000).

All three species formed distinctive groups, although the AH population of C. acuminata var. acuminata was intermixed with C. yunnanensis. Only the G9 population of C. acuminata var. tenuifolia formed a distinct group apart from all the other groups. In all analyses, C. acuminata var. acuminata appeared as the closest relative of C. yunnanensis, followed at some distance by C. lowreyana and, further away, C. acuminata var. tenuifolia.

This result is of significance to Camptotheca breeders currently engaged in the introgression of disease resistances and other useful traits from C. yunnanensis into C. acuminata var. acuminata. Strong interspecific crossing

barriers exist between C. lowreyana and C. yunnanensis. This is largely because of the geographical isolation of the two species. This is reflected in the genetic distance between them. Thus, C. lowreyana and C. yunnanensis should be separated as a distinct species.

RAPD analysis has some drawbacks, however. The alleles detected are general dominant, meaning that heterozygotes cannot be unambiguously identified during screening. Also, RAPDs will underestimate the amount of genetic variation at some loci because many different alleles can be grouped together in the null class (McDermott 1993).

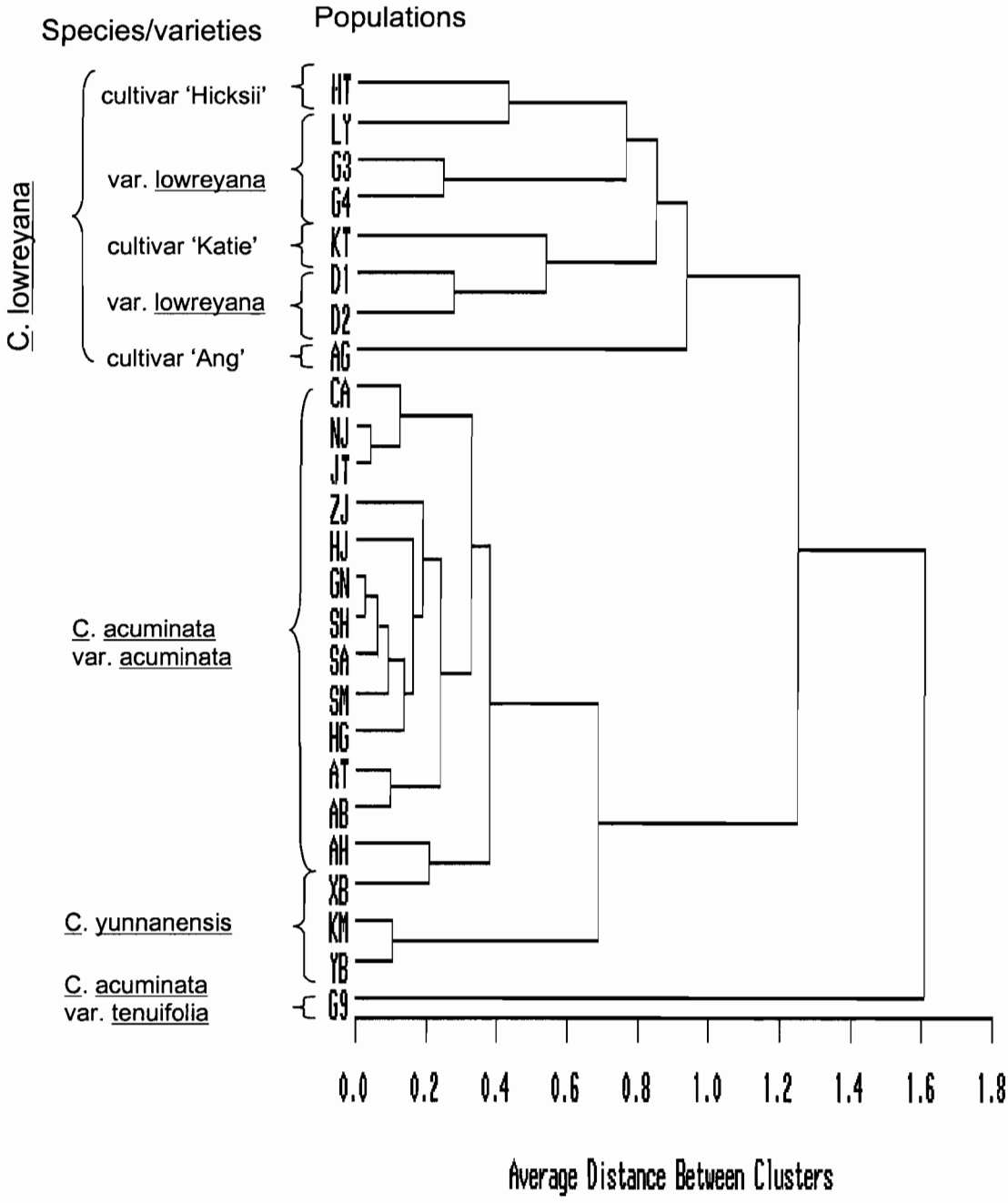


Figure 6. Dendrogram generated from RAPD DNA markers of 25 populations of Camptotheca.

Camptothecin Variation

Camptothecin Variation within and among Populations

Different varieties of Camptotheca have significant variations in leaf CPT concentration, but all can be used as CPT sources (Table 9). Obviously, however, variation in CPT content is greater among species than within species. Camptotheca lowreyana, particularly its cultivars, have higher CPT content than other taxa. Camptotheca acuminata has lower CPT content but less variation within species than the other two species. Young and old leaves show the same results. The CPT data of populations are consistent with the phenotypic variations. For example, among all varieties of Camptotheca, C. lowreyana 'Hicksii' has the highest CPT content, the largest glandular trichomes and the highest density of trichomes on the leaf surface. Camptotheca lowreyana has greater phenotypic variations among populations and the CPT variations among populations is also greater, while C. acuminata has less variation in both phenotypic traits and CPT content among populations.

More importantly, CPT data of the populations is consistent with genetic variation analysis. Species with greater genetic diversity have greater CPT variations among populations. This result suggests that CPT variation is mainly determined genetically under the same undisturbed growth conditions.

Table 9. CPT concentrations in leaves of Camptotheca (on the basis of fresh weight).

Species/Variety	Population	Young Leaf CPT Concentration (% ± s.d.)	Old Leaf CPT Concentration (% ± s.d.)
C. acuminata	NJ	0.0538± 0.0044	0.0107± 0.0020
	AH	0.0526± 0.0046	N/A
	AT	0.0672± 0.0055	N/A
	AB	0.0541± 0.0087	N/A
C. acuminata var. tenuifolia	G9	0.0776± 0.0094	N/A
C. yunnanensis	KM	0.0998± 0.0117	N/A
	YB	0.0576± 0.0104	0.0131± 0.0022
	XB	0.0765± 0.0269	N/A
C. lowreyana	LY	0.0869± 0.0071	0.0202± 0.0032
C. lowreyana 'Katie'	KT	0.1062± 0.0114	0.0217± 0.0017
C. lowreyana 'Hicksii'	HT	0.1230± 0.0077	0.0263± 0.0028

Note: young leaves: <1 week old; old leaves: 8-10 weeks.

Camptothecin Variation within Plant

Our analysis showed that CPT variation is very significant within the plant (Table 10). For young tissues, leaves have the highest CPT contents (leaves:stems:roots:fruits =30:6:1:13). For old tissues, fruits have the highest CPT contents (leaves:stems:roots:fruits =2:1:1:10). Leaves and stem bark, as photosynthesis sites, contain higher CPT contents in young tissues than old ones (young leaves:old leaves=5:1; young stem bark:old stem bark=2:1). In contrast, however, 'sink' tissues such as wood, roots, and fruits show different patterns (young wood:old wood=1:1; young roots:old roots=1:3; young fruits:old fruits=1:2).

Previous studies of CPT yield in Camptotheca by other authors are contradictory, and all are limited to C. acuminata since the plant materials of other species and varieties were not available to the researchers. Most studies showed that almost all parts of C. acuminata could yield CPTs with concentrations ranging from 0.004% to 0.400% of dry weight (Hsu et al., 1977a). It was found that the content of CPT in different parts of C. acuminata are at an average rate of 5:10:5:2:15 for roots:root bark:stem bark:stems:fruits (Hsu et al., 1977b). Fruits have the highest CPT yield according to these authors. This view has been widely accepted and thus fruits are commonly used for CPT extraction in China (Li et al., 2000).

Table 10. CPT distribution in different tissues of C. acuminata (mean ± s.d.) (on the basis of fresh weight).

		Young Tissue	Intermediate Tissue	Old Tissue
Leaf	Definition	< 1 week old	1-4 week old	> 4 week old
	CPT %	0.0514± 0.0087	0.0245± 0.0014	0.0102± 0.0017
Stem	Definition	< 4 week old	= 2 year old	= 5 year old
	CPT %	0.0106± 0.0031	0.0080± 0.0004	0.0065± 0.0008
Stem Wood	Definition	< 1 year old	= 2 year old	= 5 years old
	CPT %	0.0066± 0.00014	0.0031± 0.0001	0.0057± 0.0003
Stem Pith	Definition	< 1 year old	------	------
	CPT %	0.0143± 0.0005	------	------
Stem Bark	Definition	< 1 year old	= 2 year old	= 5 years old
	CPT %	0.0138 ±0.0026	0.0196±0.0005	0.0101± 0.0031
Flower/Fruit	Definition	< 1 week old (flower)	= 8 week old (fruit)	= 16 week old (fruit)
	CPT %	0.0228± 0.0028	0.0113± 0.0004	0.0506± 0.0029
Root	Definition	< 4 weeks	------	> 4 weeks
	CPT %	0.0017± 0.0005	------	0.0053± 0.0011

The fact that young leaves have higher yield of CPT could explain the disparity among the results of different investigators. Although none of the studies had detailed descriptions of leaf materials used, it is likely that some authors obtained positive results on CPT yield in leaves because young leaves were used in the analysis (Wall et al., 1966; Hsu et al., 1977b; Cao et al., 1992; Lopez-Meyer et al., 1994; Li 1997; Li et al., 2000). The negative results of other researchers might have been caused by the use of old leaves in the tests

(Perdue et al., 1970; Liu and Adams 1996). In addition, various authors defined "young leaves" differently. For example, some authors defined "young" leaves as those terminals on the stem (Liu and Adams 1996). However, many terminal leaves are not necessarily young in age, because many stems stop growth in the late stage of the growing season. For this study, young leaves were defined as those newly spread (mostly less than one week old). In some cases (e.g., later growth or during the drought season when the plant may cease growing in some tissues), terminal leaves on some stems are not necessarily young in age. It is also true that stems on the upper trunk are not necessarily young relative to lower stems. Consequently, no significant difference between new and old leaves was detected by researchers when the plant materials used were collected in November, when most terminal leaves are not newly spread (Liu and Adams 1996). The presence of higher CPT concentration in young leaves also indicates that the leaves which poisoned goats (Cao et al., 1992) must have been young leaves, which are more susceptible to predation.

Camptothecin Variation with Season

Unlike some previous reports of CPT concentration declining in leaves by 11% each month from April to October (Liu et al., 1998), the present study found CPT content of intact young tissues to be lowest in March and April (0.0138-0.0152%) and highest in June (0.0538%), with intermediate level in other months (0.0219-0.0320%) in C. acuminata (Figure 7). The monthly variation in CPT content was even greater than genetic variation (among populations). However,

plants usually have higher biomass growth from May to August and thus produce substantial CPT yield from the harvest of intact young tissues during this period. The data did not indicate that CPT content had a negative relationship with biomass growth.

Flower/fruit tissues also displayed a significant change in CPT content with season (Figure 8). CPT contents in mature fruits were 2-3 times those in young fruits (flowers). Obviously, CPT content in both vegetative and reproductive tissues experienced significant seasonal changes.

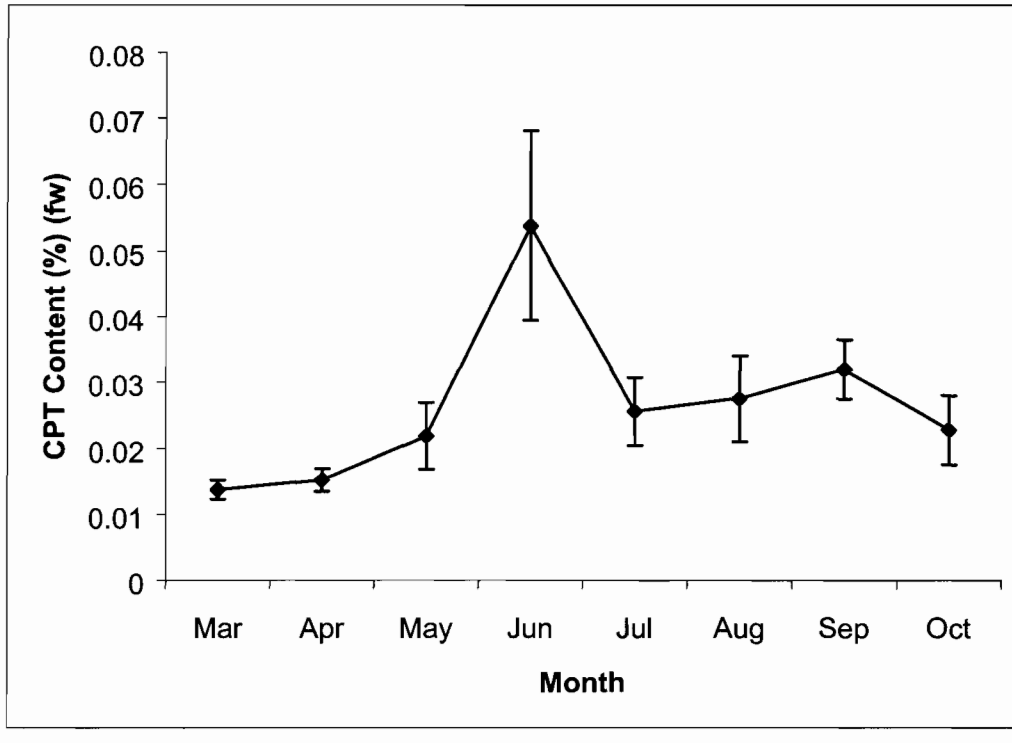


Figure 7. Monthly change of CPT content in intact young tissues of C. acuminata.

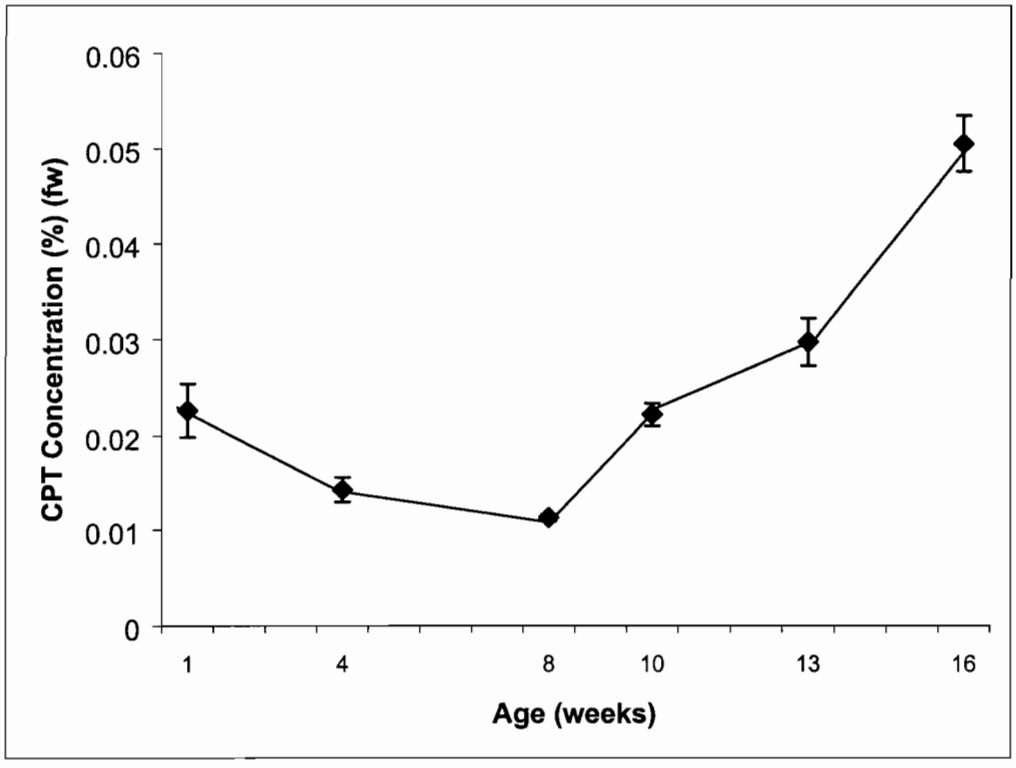


Figure 8. Fruit CPT content change over different development stages of C. acuminata. (First two weeks—flowers and rest of the weeks—fruits).

Camptothecin Variation with Age

Previously, some authors stated that CPT concentration in leaves of C. acuminata decreased with tree age significantly: 16 times lower in 4-year-old trees than in 2-year-old trees (Liu et al., 1998). However, our data do not support this hypothesis (Figure 9). The replicated studies in different months show the same result: tree age does not show a significant effect on CPT content of young leaves (May: $F_{3,10} = 1.48$, $P < 0.05$; July: $F_{6,14} = 0.77$, $P < 0.05$). Our previous

analysis indicated that the CPT concentration of young leaves is positively correlated to the CPT yield of intact young tissues. Thus, it is clear that tree age is not a factor directly influencing CPT yield of young tissues, at least from one to eight years. However, the young leaves collected in July 2000 were significantly higher in CPT concentration than those collected in May 1999. This difference was attributed to seasonal changes in CPT concentrations rather than yearly variation.

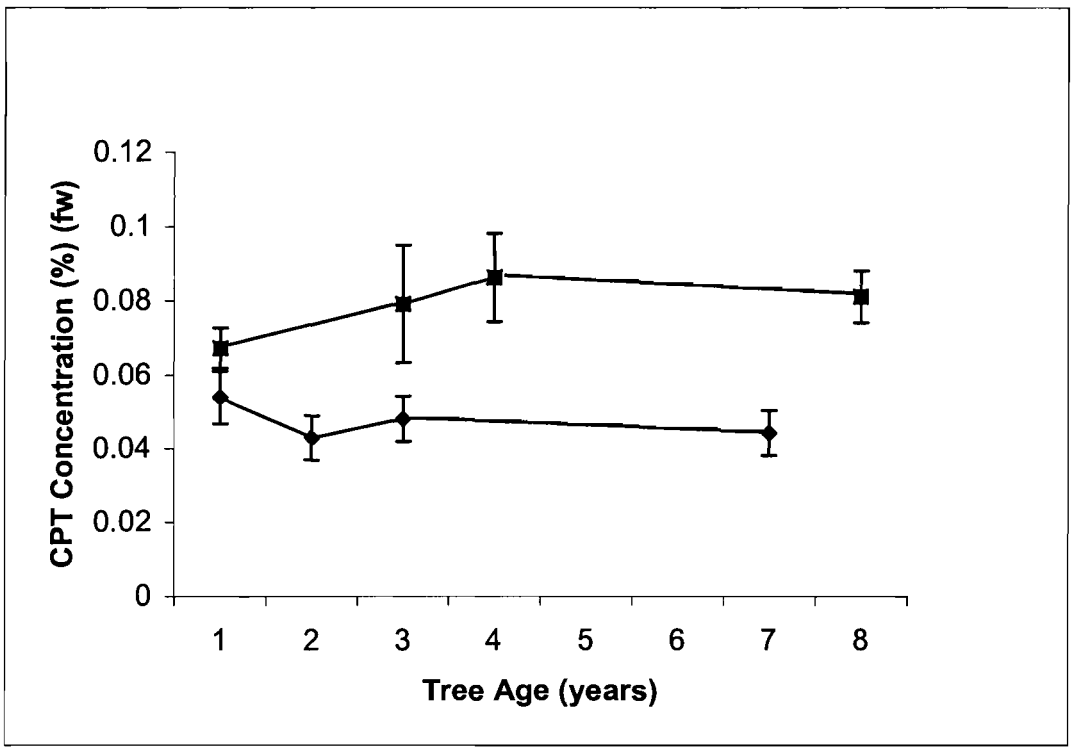


Figure 9. Variation in CPT content of young leaves with plant age in C. acuminata (♦ May; ■ July)

Camptothecin Variation With Stress

The CPT concentration of intact young tissue of C. acuminata was significantly increased by T-pruning treatments. Treatment I increased CPT content by 113.9%, and the treatment II increased CPT content by 166.2% as compared to the control. Average annual biomass yield of intact young tissue was increased by 187% by T-pruning treatment II (Figure 10). This induction of CPT is likely the result of the effective control of auxin levels in plants by pruning the shoot tips.

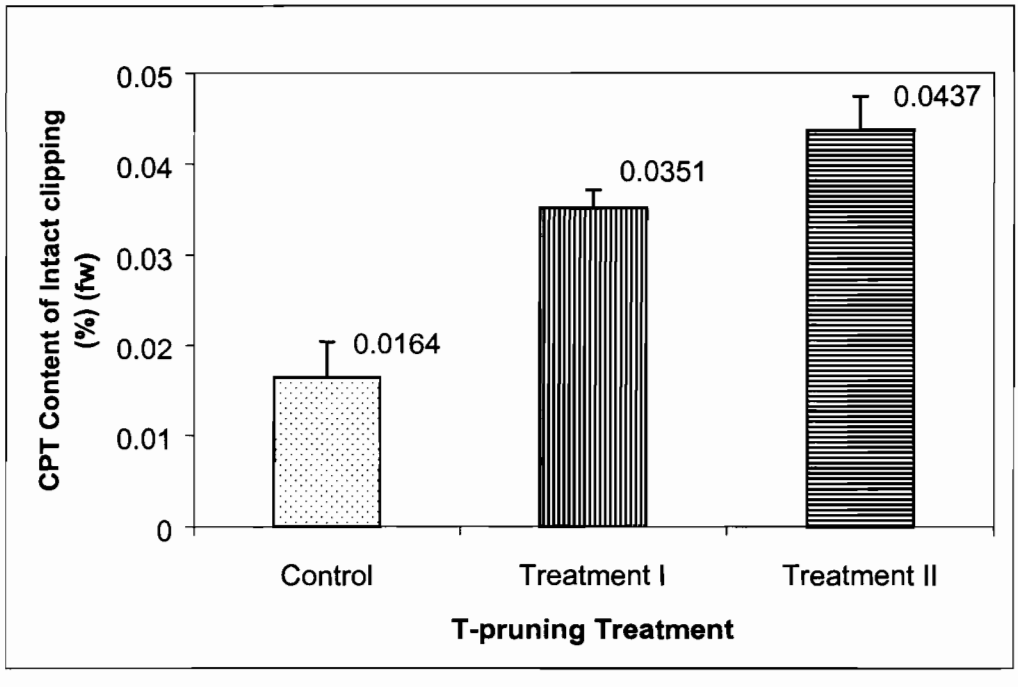


Figure 10. Effects of T-pruning treatments on CPT contents (%) of intact young tissues of C. acuminata (mean ± s.d.).

CONCLUSIONS

RAPD markers provide a powerful tool for the identification of some populations (particularly cultivars) and the detection of genetic variation within Camptotheca. The genetic diversity analysis provides a basis for strategy development for both conservation and management of these endangered anticancer trees. Three primers (OPA02, OPA03, and OPA04), generating 44 polymorphic bands, were able to discriminate among 25 Camptotheca populations. The band size varied from 268-4,411bp, with an average of 15 bands/primer. Of these populations, three can be distinguished by their unique bands, respectively: Cultivar 'Katie' (KT) (presence of OPA03-2423), the HJ population of C. acuminata (presence of OPA04-268), and cultivar 'Ang' (AG) (absence of OPA03-1460).

Camptotheca has relatively low gene diversity within population compared with other tree species. Population differentiation of Camptotheca was found to be higher than in other species with similar breeding systems. It appears that fragmentation has caused gene flow to become low enough for factors such as genetic drift and possible inbreeding depression to cause this differentiation. All populations were therefore distinctive genetically and each should be considered as a management unit. The high level of genetic structure among populations indicates differentiation due to founder events and/or genetic drift coupled with

limited migration. Therefore, a conservation approach to conserving these populations is recommended.

Cluster analysis of the genetic distance values and a dendrogram generated from the RAPD markers are consistent with the phenotypic data and both support the current taxonomic treatment of Camptotheca (Li 1997, 2001; Li et al., 2000). Camptotheca acuminata var. acuminata appeared as the closest relative of C. yunnanensis, followed at some distance by C. lowreyana and, further away, C. acuminata var. tenuifolia. Strong interspecific crossing barriers exist between C. lowreyana and C. yunnanensis due to geographical isolation.

The CPT data of Camptotheca are consistent with the phenotypic and genetic variation analysis. The results show that CPT variation in Camptotheca is mainly determined genetically under the same undisturbed growth conditions. Variation in leaf CPT content of Camptotheca is greater among species than within species. Although it has been widely planted in southern China and many other locations in the world, C. acuminata has relatively low CPT contents and less variation among populations. Considering both low genetic diversity and low CPT yield, the species is not the optimum candidate for plantation development for CPT production. In contract, C. lowreyana should be considered as a management target in both CPT production and germplasm conservation because the species not only has higher genetic diversity but also has higher CPT concentrations than the other taxa.

CPT content is highest in young leaves, with higher levels in mature fruits than other old tissues. Young photosynthetic leaves and stems contain higher CPT contents than old ones, but 'sink' tissues such as wood, roots, and fruits show different patterns. CPT content also shows a great seasonal change, but is not influenced by tree age. Preservation and treatment methods influence CPT extraction. CPT is better preserved in fresh or freeze-dry than air or oven-dry tissues.

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APPENDIX

Appendix A. Survey of 46 RAPD markers in 13 populations of C. acuminata, 9 of C. lowreyana, and 3 of C. yunnanensis

Cultivars / Primers	HT	KT	AG	D1	D2	G3	G4	G9	LY	CA	NJ	ZJ	AH	JT	HJ	GN	HG	SM	SH	SA	AT	AB	XB	KM	YB
OPA02-3015	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	*
OPA02-2500	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	*
OPA02-2400	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
OPA02-2200	+	+	*	+	+	-	-	+	-	+	+	+	+	+	+	+	*	+	+	+	+	+	+	+	+
OPA02-1750	-	*	*	*	-	-	-	+	-	+	+	+	+	+	+	+	*	+	+	*	+	+	+	+	+
OPA02-1500	+	*	*	+	*	*	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
OPA02-1300	+	-	*	+	-	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
OPA02-1200	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	*
OPA02-1080	-	+	+	-	*	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
OPA02-960	+	*	+	+	*	*	*	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
OPA02-860	+	+	+	+	+	+	+	+	*	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
OPA02-700	+	*	+	+	+	+	+	+	*	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
OPA02-560	+	+	+	+	+	+	*	+	*	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
OPA02-450	*	*	+	+	+	+	*	+	*	+	+	+	+	+	+	+	+	+	+	+	*	*	+	+	*
OPA02-345	-	-	-	-	-	-	*	+	-	+	+	+	+	+	*	*	-	*	-	-	-	-	*	*	*
OPA02-271	-	-	-	-	-	-	*	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
OPA03-2423	-	[redacted]	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
OPA03-2000	-	+	-	*	-	-	-	+	-	*	*	*	*	-	-	-	-	-	*	-	+	*	+	+	+
OPA03-1770	-	-	+	*	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
OPA03-1600	-	-	-	-	-	-	-	-	-	+	+	*	+	+	+	+	+	+	+	+	+	+	+	+	+
OPA03-1460	+	+	[redacted]	+	+	*	*	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
OPA03-1210	+	+	+	+	+	+	+	+	+	*	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
[redacted]	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
OPA03-910	-	+	-	+	+	-	-	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
OPA03-800	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	*
OPA03-700	+	+	+	+	+	+	+	+	+	+	*	+	+	+	+	+	+	+	+	+	+	+	+	+	+
OPA03-530	-	-	+	-	-	*	-	+	-	*	*	+	+	-	+	+	+	+	+	+	+	+	+	+	+
[redacted]	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
OPA03-348	-	-	-	-	-	-	-	-	-	*	-	-	-	-	-	-	-	-	*	-	-	*	-	-	-

Appendix A. (continued)

Cultivars / Primers	HT	KT	AG	D1	D2	G3	G4	G9	LY	CA	NJ	ZJ	AH	JT	HJ	GN	HG	SM	SH	SA	AT	AB	XB	KM	YB
OPA03-270	-	-	-	-	-	-	-	-	-	*	-	-	-	-	-	-	-	-	*	-	-	*	-	-	-
OPA04-4411	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	*	-	-	-
OPA04-2500	-	+	+	+	+	+	-	+	-	-	*	*	-	-	*	-	-	-	-	-	-	*	-	-	-
OPA04-1800	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	*	-
OPA04-1650	-	*	*	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
OPA04-1502	+	*	+	-	-	-	-	-	+	+	+	+	-	+	+	+	+	*	+	+	+	+	-	-	-
OPA04-1360	-	-	-	-	-	-	-	-	-	-	*	+	+	*	+	+	+	+	+	+	+	*	*	-	-
OPA04-1180	-	-	-	-	-	*	-	+	-	-	-	*	-	-	-	-	-	-	-	-	-	*	-	-	-
OPA04-1050	-	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-	*	-	-	-	-	-	*	-
OPA04-880	-	-	-	-	-	-	-	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	*	*	-
OPA04-780	+	+	+	+	-	+	+	+	+	+	*	+	+	-	+	+	+	*	+	*	+	+	+	+	+
OPA04-670	-	-	+	-	-	*	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
OPA04-560	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	*	-
OPA04-450	-	*	*	-	-	*	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	*	-	-	-
OPA04-370	-	-	-	-	-	*	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
OPA04-290	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+	-	*	*	*	-
OPA04-268	-	-	-	-	-	-	-	-	-	-	-	-	-	-	[redacted]	-	-	-	-	-	-	-	-	-	-

"+" means homo presence; "-" means absence; "*" means polymorphic presence

Appendix B. Summary allele frequency data for 44 polymorphic loci among three species of Camptotheca, two varieties of C. acuminata, three cultivars and one varieties of C. lowreyana

Locus/allele	Within species			All species	C. acuminata		C. lowreyana			
	C. acuminata	C. lowreyana	C. yunnanensis		var. acuminata	var. tenifolia	var. lowreyana	'HT'	'KT'	'AG'
OPA02-3015 a	0.9661	1.0000	0.9588	0.9739	1.0000	0.0000	1.0000	1.0000	1.0000	1.0000
b	0.0339	0.0000	0.0412	0.0261	0.0000	1.0000	0.0000	0.0000	0.0000	0.0000
OPA02-2500 a	0.9661	1.0000	0.9588	0.9739	1.0000	0.0000	1.0000	1.0000	1.0000	1.0000
b	0.0339	0.0000	0.0412	0.0261	0.0000	1.0000	0.0000	0.0000	0.0000	0.0000
OPA02-2400 a	1.0000	0.3200	1.0000	0.8247	1.0000	1.0000	0.5714	0.0000	0.0000	0.0000
b	0.0000	0.6800	0.0000	0.1753	0.0000	0.0000	0.4286	1.0000	1.0000	1.0000
OPA02-2200 a	0.0656	0.4094	0.0000	0.1455	0.0679	0.0000	0.5714	0.0000	0.0000	0.4472
b	0.9344	0.5906	1.0000	0.8545	0.9321	1.0000	0.4286	1.0000	1.0000	0.5528
OPA02-1750 a	0.0995	0.9115	0.0000	0.2955	0.1030	0.0000	0.9607	1.0000	0.8660	0.7746
b	0.9005	0.0885	1.0000	0.7045	0.8970	1.0000	0.0393	0.0000	0.1340	0.2254
OPA02-1500 a	0.9661	0.5684	1.0000	0.8682	1.0000	0.0000	0.6533	0.0000	0.7071	0.4472
b	0.0339	0.4316	0.0000	0.1318	0.0000	1.0000	0.3467	1.0000	0.2929	0.5528
OPA02-1300 a	0.0000	0.6989	0.0000	0.1801	0.0000	0.0000	0.6429	0.0000	1.0000	0.8944
b	1.0000	0.3011	1.0000	0.8199	1.0000	1.0000	0.3571	1.0000	0.0000	0.1056
OPA02-1200 a	0.0000	0.0000	0.1538	0.0206	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
b	1.0000	1.0000	0.8462	0.9794	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000
OPA02-1080 a	1.0000	0.6180	1.0000	0.9015	1.0000	1.0000	0.9607	1.0000	0.0000	0.0000
b	0.0000	0.3820	0.0000	0.0985	0.0000	0.0000	0.0393	0.0000	1.0000	1.0000
OPA02-960 a	0.0000	0.2859	0.0000	0.0737	0.0000	0.0000	0.3676	0.0000	0.5000	0.0000
b	1.0000	0.7141	1.0000	0.9263	1.0000	1.0000	0.6324	1.0000	0.5000	1.0000
OPA02-860 a	0.0000	0.0566	0.0000	0.0146	0.0000	0.0000	0.1010	0.0000	0.0000	0.0000
b	1.0000	0.9436	1.0000	0.9854	1.0000	1.0000	0.8990	1.0000	1.0000	1.0000
OPA02-700 a	0.0000	0.1951	0.0000	0.0503	0.0000	0.0000	0.1010	0.0000	0.8660	0.0000
b	1.0000	0.8049	1.0000	0.9497	1.0000	1.0000	0.8990	1.0000	0.1340	1.0000
OPA02-560 a	0.0000	0.1366	0.0000	0.0352	0.0000	0.0000	0.2439	0.0000	0.0000	0.0000
b	1.0000	0.8634	1.0000	0.9648	1.0000	1.0000	0.7561	1.0000	1.0000	1.0000
OPA02-450 a	0.0915	0.3063	0.2176	0.1637	0.0947	0.0000	0.2439	0.7071	0.7071	0.0000
b	0.9085	0.6937	0.7824	0.8363	0.9053	1.0000	0.7561	0.2929	0.2929	1.0000
OPA02-345 a	0.5578	0.9786	0.8309	0.7028	0.5773	0.0000	0.9617	1.0000	1.0000	1.0000
b	0.4422	0.0214	0.1691	0.2972	0.4227	1.0000	0.0383	0.0000	0.0000	0.0000

Appendix B. (continued)

Locus/allele	Within species			All species	C. acuminata		C. lowreyana			
	C. acuminata	C. lowreyana	C. yunnanensis		var. acuminata	var. tenifolia	var. lowreyana	'HT'	'KT'	'AG'
OPA02-271 a	0.9661	0.9786	1.0000	0.9739	1.0000	0.0000	0.9617	1.0000	1.0000	1.0000
b	0.0339	0.0214	0.0000	0.0261	0.0000	1.0000	0.0383	0.0000	0.0000	0.0000
OPA03-2423 a	1.0000	0.8400	1.0000	0.9588	1.0000	1.0000	1.0000	1.0000	0.0000	1.0000
b	0.0000	0.1600	0.0000	0.0412	0.0000	0.0000	0.0000	0.0000	1.0000	0.0000
OPA03-2000 a	0.7469	0.7893	0.0000	0.6577	0.7731	0.0000	0.9094	1.0000	0.0000	1.0000
b	0.2531	0.2107	1.0000	0.3423	0.2269	1.0000	0.0906	0.0000	1.0000	0.0000
OPA03-1770 a	0.9661	0.7780	1.0000	0.9222	1.0000	0.0000	0.9607	1.0000	1.0000	0.0000
b	0.0339	0.2220	0.0000	0.0778	0.0000	1.0000	0.0393	0.0000	0.0000	1.0000
OPA03-1600 a	0.0818	1.0000	0.0000	0.3075	0.0496	1.0000	1.0000	1.0000	1.0000	1.0000
b	0.9182	0.0000	1.0000	0.6925	0.9504	0.0000	0.0000	0.0000	0.0000	0.0000
OPA03-1460 a	0.0000	0.3697	0.0000	0.0953	0.0000	0.0000	0.3030	0.0000	0.0000	1.0000
b	1.0000	0.6303	1.0000	0.9047	1.0000	1.0000	0.6970	1.0000	1.0000	0.0000
OPA03-1210 a	0.0379	0.0000	0.0000	0.0231	0.0392	0.0000	0.0000	0.0000	0.0000	0.0000
b	0.9621	1.0000	1.0000	0.9769	0.9608	1.0000	1.0000	1.0000	1.0000	1.0000
OPA03-910 a	0.0339	0.6000	0.0000	0.1753	0.0000	1.0000	0.5714	1.0000	0.0000	1.0000
b	0.9661	0.4000	1.0000	0.8247	1.0000	0.0000	0.4286	0.0000	1.0000	0.0000
OPA03-800 a	0.0000	0.0000	0.1538	0.0206	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
b	1.0000	1.0000	0.8462	0.9794	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000
OPA03-700 a	0.0397	0.0000	0.0000	0.0231	0.0392	0.0000	0.0000	0.0000	0.0000	0.0000
b	0.9621	1.0000	1.0000	0.9769	0.9608	1.0000	1.0000	1.0000	1.0000	1.0000
OPA03-530 a	0.1633	0.7766	0.0000	0.2995	0.1690	0.0000	0.9582	1.0000	1.0000	0.0000
b	0.8376	0.2234	1.0000	0.7005	0.8310	1.0000	0.0418	0.0000	0.0000	1.0000
OPA03-348 a	0.9626	1.0000	1.0000	0.9773	0.9613	1.0000	1.0000	1.0000	1.0000	1.0000
b	0.0374	0.0000	0.0000	0.0227	0.0387	0.0000	0.0000	0.0000	0.0000	0.0000
OPA03-270 a	0.9728	1.0000	1.0000	0.9834	0.9718	1.0000	1.0000	1.0000	1.0000	1.0000
b	0.0272	0.0000	0.0000	0.0166	0.0282	0.0000	0.0000	0.0000	0.0000	0.0000
OPA04-4411 a	0.9911	1.0000	1.0000	0.9946	0.9907	1.0000	1.0000	1.0000	1.0000	1.0000
b	0.0089	0.0000	0.0000	0.0054	0.0093	0.0000	0.0000	0.0000	0.0000	0.0000
OPA04-2500 a	0.8997	0.3200	1.0000	0.7637	0.9313	0.0000	0.4286	1.0000	0.0000	0.0000
b	0.1003	0.6800	0.0000	0.2363	0.0687	1.0000	0.5714	0.0000	1.0000	1.0000

Appendix B. (continued)

Locus/allele		Within species			All species	C. acuminata		C. lowreyana			
		C. acuminata	C. lowreyana	C. yunnanensis		var. acuminata	var. tenifolia	var. lowreyana	'HT'	'KT'	'AG'
OPA04-1800	a	0.0000	0.0000	0.6517	0.0873	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
	b	1.0000	1.0000	0.3483	0.9127	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000
OPA04-1650	a	1.0000	0.9574	1.0000	0.9890	1.0000	1.0000	1.0000	1.0000	0.8660	0.8944
	b	0.0000	0.0426	0.0000	0.0110	0.0000	0.0000	0.0000	0.0000	0.1340	0.1056
OPA04-1502	a	0.1673	0.5600	1.0000	0.3801	0.1381	1.0000	0.8571	0.0000	0.5000	0.0000
	b	0.8327	0.4400	0.0000	0.6199	0.8619	0.0000	0.1429	1.0000	0.5000	1.0000
OPA04-1360	a	0.2498	1.0000	0.9099	0.5316	0.2235	1.0000	1.0000	1.0000	1.0000	1.0000
	b	0.7502	0.0000	0.0901	0.4684	0.7765	0.0000	0.0000	0.0000	0.0000	0.0000
OPA04-1180	a	0.9481	0.9766	1.0000	0.9624	0.9813	0.0000	0.9582	1.0000	1.0000	1.0000
	b	0.0519	0.0234	0.0000	0.0376	0.0187	1.0000	0.0418	0.0000	0.0000	0.0000
OPA04-1050	a	0.9470	0.1600	0.9594	0.7458	0.9802	0.0000	0.1429	1.0000	0.0000	0.0000
	b	0.0530	0.8400	0.0406	0.2542	0.0198	1.0000	0.8571	0.0000	1.0000	1.0000
OPA04-880	a	0.0339	1.0000	0.9182	0.4014	0.0000	1.0000	1.0000	1.0000	1.0000	1.0000
	b	0.9661	0.0000	0.0818	0.5986	1.0000	0.0000	0.0000	0.0000	0.0000	0.0000
OPA04-780	a	0.1593	0.1200	0.0000	0.1278	0.1649	0.0000	0.2143	0.0000	0.0000	0.0000
	b	0.8407	0.8800	1.0000	0.8722	0.8351	1.0000	0.7857	1.0000	1.0000	1.0000
OPA04-670	a	1.0000	0.7766	1.0000	0.9424	1.0000	1.0000	0.9582	1.0000	1.0000	0.0000
	b	0.0000	0.2234	0.0000	0.0576	0.0000	0.0000	0.0418	0.0000	0.0000	1.0000
OPA04-560	a	0.0000	0.0000	0.6056	0.0812	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
	b	1.0000	1.0000	0.3944	0.9188	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000
OPA04-450	a	0.9572	0.6846	1.0000	0.8926	0.9907	0.0000	0.6724	1.0000	0.8660	0.4472
	b	0.0428	0.3154	0.0000	0.1074	0.0093	1.0000	0.3276	0.0000	0.1340	0.5528
OPA04-370	a	1.0000	0.9766	1.0000	0.9940	1.0000	1.0000	0.9582	1.0000	1.0000	1.0000
	b	0.0000	0.0234	0.0000	0.0060	0.0000	0.0000	0.0418	0.0000	0.0000	0.0000
OPA04-290	a	0.2061	0.0000	0.9182	0.2484	0.2134	0.0000	0.0000	0.0000	0.0000	0.0000
	b	0.7939	1.0000	0.0818	0.7516	0.7866	1.0000	1.0000	1.0000	1.0000	1.0000
OPA04-268	a	0.9153	1.0000	1.0000	0.9485	0.9123	1.0000	1.0000	1.0000	1.0000	1.0000
	b	0.0847	0.0000	0.0000	0.0515	0.0877	0.0000	0.0000	0.0000	0.0000	0.0000

Appendix C1. Gene diversity statistics for 25 Camptotheca populations examined with 44 RAPD polymorphic loci detected with three primers

Locus	H_T	H_S	D_{ST}	G_{ST}	Nm
OPA02-3015	0.0866	0.0093	0.0773	0.8928	0.0600
OPA02-2500	0.0866	0.0093	0.0773	0.8928	0.0600
OPA02-2400	0.3200	0.0000	0.3200	1.0000	0.0000
OPA02-2200	0.2807	0.0337	0.2470	0.8798	0.0683
OPA02-1750	0.4546	0.0692	0.3854	0.8478	0.0898
OPA02-1500	0.2983	0.0724	0.2259	0.7572	0.1603
OPA02-1300	0.3149	0.0076	0.3073	0.9760	0.0123
OPA02-1200	0.0392	0.0200	0.0192	0.4898	0.5208
OPA02-1080	0.1594	0.0120	0.1474	0.9248	0.0406
OPA02-960	0.1661	0.0761	0.0900	0.5418	0.4229
OPA02-860	0.0550	0.0166	0.0384	0.6986	0.2157
OPA02-700	0.1179	0.0259	0.0921	0.7808	0.1404
OPA02-560	0.0919	0.0366	0.0553	0.6021	0.3304
OPA02-450	0.2905	0.1246	0.1658	0.5709	0.3759
OPA02-345	0.4183	0.0883	0.2803	0.7888	0.1339
OPA02-271	0.0866	0.0093	0.0773	0.8928	0.0600
OPA03-2423	0.0768	0.0000	0.0768	1.0000	0.0000
OPA03-2000	0.4445	0.1157	0.0253	0.7397	0.1760
OPA03-1770	0.1594	0.0120	0.1474	0.9248	0.0406
OPA03-1600	0.4750	0.0166	0.4584	0.9651	0.0181
OPA03-1460	0.1745	0.0331	0.1414	0.8101	0.1172
OPA03-1210	0.0351	0.0198	0.0164	0.4671	0.6438
OPA03-910	0.3648	0.0000	0.3648	1.0000	0.0000
OPA03-800	0.0392	0.0200	0.0192	0.4898	0.5208
OPA03-700	0.0351	0.0198	0.0153	0.4371	0.6438
OPA03-530	0.4659	0.0427	0.0427	0.9083	0.0505
OPA03-348	0.0403	0.0335	0.0068	0.1688	2.4627
OPA03-270	0.0311	0.0271	0.0040	0.1280	3.4049
OPA04-4411	0.0084	0.0076	0.0009	0.1018	4.4126
OPA04-2500	0.3964	0.0512	0.3452	0.8709	0.0741
OPA04-1800	0.1401	0.0076	0.1325	0.9461	0.0285
OPA04-1650	0.0190	0.0168	0.0021	0.1130	3.9264
OPA04-1502	0.4842	0.0340	0.4502	0.9298	0.0377

Appendix C1. (continued)

Locus	H_T	H_S	D_{ST}	G_{ST}	Nm
OPA04-1360	0.4848	0.0703	0.4145	0.8549	0.0848
OPA04-1180	0.1151	0.0334	0.0817	0.7097	0.2045
OPA04-1050	0.4145	0.0215	0.3930	0.9481	0.0274
OPA04-880	0.4982	0.0168	0.4814	0.9662	0.0175
OPA04-780	0.2454	0.0584	0.1870	0.7621	0.1561
OPA04-670	0.0981	0.0166	0.0815	0.8311	0.1016
OPA04-560	0.1319	0.0140	0.1179	0.8941	0.0592
OPA04-450	0.2164	0.0532	0.1632	0.7542	0.1630
OPA04-370	0.0232	0.0166	0.0066	0.2845	1.2574
OPA04-290	0.3384	0.0354	0.3030	0.8953	0.0585
OPA04-268	0.0768	0.0000	0.0768	1.0000	0.0000
Mean	0.2113	0.0319	0.1794	0.8490	0.0889

H_T is total variation in all populations, H_S is the average gene diversity found within populations, D_{ST} is the average gene diversity among populations, G_{ST}, equivalent to D_{ST}/H_T, is the proportion of total gene diversity due to differences among populations, and Nm is the estimate of gene flow from Gst, Nm = 0.5(1-Gst)/Gst.

Appendix C2. Genetic Diversity estimates for Camptotheca lowreyana, C. acuminata, and C. yunnanensis.

Locus	H_T	H_S	D_{ST}	G_{ST}	Nm
			C. lowreyana		
OPA02-3015	0.0000	0.0000	****	****	****
OPA02-2500	0.0000	0.0000	****	****	****
OPA02-2400	0.4688	0.0000	0.4688	1.0000	0.0000
OPA02-2200	0.4905	0.0618	0.4287	0.8740	0.0721
OPA02-1750	0.1265	0.1101	0.0164	0.1296	3.3576
OPA02-1500	0.4940	0.2264	0.2676	0.5417	0.4229
OPA02-1300	0.4750	0.0236	0.4514	0.9503	0.0261
OPA02-1200	0.0000	0.0000	0.0000	****	****
OPA02-1080	0.3969	0.0375	0.3594	0.9055	0.0521
OPA02-960	0.4080	0.2378	0.1702	0.4171	0.6983
OPA02-860	0.1612	0.0518	0.1094	0.6787	0.2367
OPA02-700	0.3159	0.0808	0.2351	0.7442	0.1718
OPA02-560	0.2562	0.1143	0.1419	0.5539	0.4025
OPA02-450	0.4406	0.2178	0.2228	0.5057	0.4889
OPA02-345	0.0329	0.0290	0.0039	0.1185	3.6938
OPA02-271	0.0329	0.0290	0.0039	0.1185	3.6938
OPA03-2423	0.2188	0.0000	0.2188	1.0000	0.0000
OPA03-2000	0.2924	0.0610	0.2314	0.7914	0.1318
OPA03-1770	0.2521	0.0375	0.2146	0.8512	0.0873
OPA03-1600	0.0000	0.0000	0.0000	****	****
OPA03-1460	0.4214	0.1036	0.3178	0.7542	0.1629
OPA03-1210	0.0000	0.0000	0.0000	****	****
OPA03-910	0.4688	0.0000	0.4688	1.0000	0.0000
OPA03-800	0.0000	0.0000	0.0000	****	****
OPA03-700	0.0000	0.0000	0.0000	****	****
OPA03-530	0.2710	0.0518	0.2192	0.8089	0.1181
OPA03-348	0.0000	0.0000	0.0000	****	****
OPA03-270	0.0000	0.0000	0.0000	****	****

Appendix C2. (continued)

Locus	H_T	H_S	D_{ST}	G_{ST}	Nm
			C. lowreyana		
OPA04-4411	0.0000	0.0000	0.0000	****	****
OPA04-2500	0.4688	0.0000	0.4688	1.0000	0.0000
OPA04-1800	0.0000	0.0000	0.0000	****	****
OPA04-1650	0.0581	0.0526	0.0055	0.0947	4.8000
OPA04-1502	0.4922	0.0625	0.4297	0.8730	0.0727
OPA04-1360	0.0000	0.0000	0.0000	****	****
OPA04-1180	0.0705	0.0518	0.0187	0.2652	1.3796
OPA04-1050	0.3750	0.0000	0.3750	1.0000	0.0000
OPA04-880	0.0000	0.0000	0.0000	****	****
OPA04-780	0.2188	0.0000	0.2188	1.0000	0.0000
OPA04-670	0.2710	0.0518	0.2192	0.8089	0.1181
OPA04-560	0.0000	0.0000	0.0000	****	****
OPA04-450	0.3724	0.1426	0.2298	0.6171	0.3102
OPA04-370	0.0705	0.0518	0.0187	0.2652	1.3796
OPA04-290	0.0000	0.0000	0.0000	****	****
OPA04-268	0.0000	0.0000	0.0000	****	****
Mean	0.1914	0.0429	0.1485	0.7760	0.1444
			C. acuminata		
OPA02-3015	0.1327	0.0000	0.1327	1.0000	0.0000
OPA02-2500	0.1327	0.0000	0.1327	1.0000	0.0000
OPA02-2400	0.0000	0.0000	0.0000	****	****
OPA02-2200	0.1045	0.0249	0.0796	0.7614	0.1567
OPA02-1750	0.1655	0.0607	0.1048	0.6335	0.2893
OPA02-1500	0.1327	0.0000	0.1327	1.0000	0.0000
OPA02-1300	0.0000	0.0000	0.0000	****	****
OPA02-1200	0.0000	0.0000	0.0000	****	****
OPA02-1080	0.0000	0.0000	0.0000	****	****
OPA02-960	0.0000	0.0000	0.0000	****	****

Appendix C2. (continued)

Locus	H_T	H_S	D_{ST}	G_{ST}	Nm
			C. acuminata		
OPA02-860	0.0000	0.0000	0.0000	****	****
OPA02-700	0.0000	0.0000	0.0000	****	****
OPA02-560	0.0000	0.0000	0.0000	****	****
OPA02-450	0.1423	0.0685	0.0738	0.5186	0.4641
OPA02-345	0.4997	0.0831	0.4166	0.8337	0.0997
OPA02-271	0.1327	0.0000	0.1327	1.0000	0.0000
OPA03-2423	0.0000	0.0000	0.0000	****	****
OPA03-2000	0.4028	0.1718	0.2310	0.5735	0.3718
OPA03-1770	0.1327	0.0000	0.1327	1.0000	0.0000
OPA03-1600	0.2141	0.0296	0.1845	0.8618	0.0802
OPA03-1460	0.0000	0.0000	0.0000	****	****
OPA03-1210	0.0618	0.0353	0.0265	0.4290	0.6656
OPA03-910	0.1327	0.0000	0.1327	1.0000	0.0000
OPA03-800	0.0000	0.0000	0.0000	****	****
OPA03-700	0.0618	0.0353	0.0265	0.4290	0.6656
OPA03-530	0.2958	0.0467	0.2491	0.8421	0.0937
OPA03-348	0.0708	0.0598	0.0110	0.1548	2.7298
OPA03-270	0.0548	0.0484	0.0064	0.1169	3.7766
OPA04-4411	0.0150	0.0135	0.0015	0.0988	4.5619
OPA04-2500	0.2252	0.0914	0.1338	0.5941	0.3417
OPA04-1800	0.0000	0.0000	0.0000	****	****
OPA04-1650	0.0000	0.0000	0.0000	****	****
OPA04-1502	0.3178	0.0249	0.2929	0.9215	0.0426
OPA04-1360	0.4064	0.0960	0.3104	0.7638	0.1546
OPA04-1180	0.1614	0.0301	0.1313	0.8137	0.1145
OPA04-1050	0.1597	0.0249	0.1348	0.8439	0.0925
OPA04-880	0.1327	0.0000	0.1327	1.0000	0.0000
OPA04-780	0.3006	0.1042	0.1964	0.6533	0.2654

Appendix C2. (continued)

Locus	H_T	H_S	D_{ST}	G_{ST}	Nm
			C. acuminata		
OPA04-670	0.0000	0.0000	0.0000	****	****
OPA04-560	0.0000	0.0000	0.0000	****	****
OPA04-450	0.1455	0.0135	0.1320	0.9073	0.0511
OPA04-370	0.0000	0.0000	0.0000	****	****
OPA04-290	0.3054	0.0332	0.2722	0.8912	0.0610
OPA04-268	0.1327	0.0000	0.1327	1.0000	0.0000
Mean	0.1176	0.0249	0.0927	0.7881	0.1344
			C. yunnanensis		
OPA02-3015	0.0853	0.0774	0.0079	0.0935	4.8481
OPA02-2500	0.0853	0.0774	0.0079	0.0935	4.8481
OPA02-2400	0.0000	0.0000	0.0000	****	****
OPA02-2200	0.0000	0.0000	0.0000	****	****
OPA02-1750	0.0000	0.0000	0.0000	****	****
OPA02-1500	0.0000	0.0000	0.0000	****	****
OPA02-1300	0.0000	0.0000	0.0000	****	****
OPA02-1200	0.2778	0.1667	0.1111	0.4000	0.7500
OPA02-1080	0.0000	0.0000	0.0000	****	****
OPA02-960	0.0000	0.0000	0.0000	****	****
OPA02-860	0.0000	0.0000	0.0000	****	****
OPA02-700	0.0000	0.0000	0.0000	****	****
OPA02-560	0.0000	0.0000	0.0000	****	****
OPA02-450	0.3603	0.1381	0.2222	0.6168	0.3107
OPA02-345	0.2748	0.2711	0.0037	0.0135	36.4850
OPA02-271	0.0000	0.0000	0.0000	****	****
OPA03-2423	0.0000	0.0000	0.0000	****	****
OPA03-2000	0.0000	0.0000	0.0000	****	****
OPA03-1770	0.0000	0.0000	0.0000	****	****
OPA03-1600	0.0000	0.0000	0.0000	****	****

Appendix C2. (continued)

Locus	H_T	H_S	D_{ST}	G_{ST}	Nm
			C. yunnanensis		
OPA03-1460	0.0000	0.0000	0.0000	****	****
OPA03-1210	0.0000	0.0000	0.0000	****	****
OPA03-910	0.0000	0.0000	0.0000	****	****
OPA03-800	0.2778	0.1667	0.1111	0.4000	0.7500
OPA03-700	0.0000	0.0000	0.0000	****	****
OPA03-530	0.0000	0.0000	0.0000	****	****
OPA03-348	0.0000	0.0000	0.0000	****	****
OPA03-270	0.0000	0.0000	0.0000	****	****
OPA04-4411	0.0000	0.0000	0.0000	****	****
OPA04-2500	0.0000	0.0000	0.0000	****	****
OPA04-1800	0.4654	0.0630	0.4024	0.8647	0.0782
OPA04-1650	0.0000	0.0000	0.0000	****	****
OPA04-1502	0.0000	0.0000	0.0000	****	****
OPA04-1360	0.1762	0.1381	0.0381	0.2164	1.8107
OPA04-1180	0.0000	0.0000	0.0000	****	****
OPA04-1050	0.0679	0.0630	0.0049	0.0729	6.3541
OPA04-880	0.1469	0.1403	0.0066	0.0452	10.5573
OPA04-780	0.0000	0.0000	0.0000	****	****
OPA04-670	0.0000	0.0000	0.0000	****	****
OPA04-560	0.4832	0.1164	0.3668	0.7591	0.1586
OPA04-450	0.0000	0.0000	0.0000	****	****
OPA04-370	0.0000	0.0000	0.0000	****	****
OPA04-290	0.1469	0.1403	0.0066	0.0452	10.5573
OPA04-268	0.0000	0.0000	0.0000	****	****
Mean	0.0647	0.0354	0.0293	0.4529	0.6040

H_T is total variation in each species, H_S is the average gene diversity within populations, D_{ST} is the average gene diversity among populations, and G_{ST}, equivalent to D_{ST}/H_T, is the proportion of total gene diversity due to differences among populations.

Appendix D. Nei's Genetic Identity and Genetic distance of all 25 populations of Camptotheca

pop ID	HT	KT	AG	D1	D2	G3	G4	G9	LY	CA	NJ	ZJ
HT	****	0.7927	0.7878	0.8903	0.8332	0.8004	0.8106	0.6689	0.8954	0.8160	0.8044	0.7982
KT	0.2323	****	0.7781	0.8658	0.8752	0.7938	0.7659	0.6304	0.7449	0.6932	0.6969	0.6910
AG	0.2386	0.2509	****	0.8026	0.7966	0.8480	0.7901	0.6969	0.7200	0.6696	0.6548	0.6885
D1	0.1162	0.1441	0.2199	****	0.9312	0.8526	0.7982	0.7546	0.7831	0.7842	0.7853	0.7764
D2	0.1825	0.1332	0.2274	0.0713	****	0.8805	0.8281	0.6611	0.7521	0.7424	0.7708	0.7311
G3	0.2226	0.2309	0.1649	0.1595	0.1273	****	0.9391	0.7131	0.8191	0.7180	0.7123	0.7175
G4	0.2100	0.2667	0.2357	0.2253	0.1887	0.0628	****	0.6791	0.8602	0.7278	0.7151	0.7101
G9	0.4021	0.4614	0.3611	0.2816	0.4139	0.3381	0.3870	****	0.6063	0.6650	0.6563	0.6982
LY	0.1105	0.2945	0.3285	0.2445	0.2849	0.1995	0.1506	0.5003	****	0.8014	0.7894	0.7835
CA	0.2034	0.3664	0.4011	0.2431	0.2979	0.3312	0.3178	0.4079	0.2214	****	0.9731	0.9475
NJ	0.2176	0.3611	0.4234	0.2417	0.2604	0.3392	0.3353	0.4211	0.2364	0.0273	****	0.9448
ZJ	0.2254	0.3696	0.3732	0.2530	0.3132	0.3320	0.3423	0.3592	0.2440	0.0539	0.0567	****
AH	0.3145	0.4319	0.4880	0.2847	0.3521	0.3750	0.3733	0.3941	0.3359	0.0931	0.0965	0.0616
JT	0.2194	0.3900	0.4439	0.2619	0.2525	0.3547	0.3339	0.4578	0.2375	0.0353	0.0104	0.0750
HJ	0.2526	0.4154	0.3981	0.2831	0.3367	0.3591	0.3787	0.4573	0.2720	0.0758	0.0795	0.0480
GN	0.2182	0.3884	0.3718	0.2606	0.3126	0.3336	0.3393	0.4400	0.2362	0.0542	0.0602	0.0286
HG	0.2078	0.3837	0.3487	0.2583	0.3015	0.2724	0.2804	0.5009	0.1813	0.0948	0.1013	0.0678
SM	0.2465	0.3822	0.3924	0.2303	0.2540	0.3016	0.3074	0.4067	0.2660	0.0766	0.0685	0.0499
SH	0.2259	0.3701	0.3827	0.2583	0.3221	0.3439	0.3516	0.4375	0.2445	0.0656	0.0724	0.0386
SA	0.2043	0.3777	0.3643	0.2518	0.2671	0.3186	0.3264	0.4769	0.2221	0.0773	0.0675	0.0508
AT	0.2629	0.3725	0.4486	0.3011	0.3839	0.4084	0.4000	0.4624	0.2825	0.1149	0.1154	0.0780
AB	0.2032	0.3352	0.3718	0.2506	0.3141	0.3325	0.3309	0.4200	0.2219	0.0670	0.0827	0.0681
XB	0.2443	0.3189	0.4047	0.2037	0.2789	0.2987	0.3053	0.3426	0.2635	0.1195	0.1340	0.1186
KM	0.2878	0.3605	0.4501	0.2393	0.3178	0.3396	0.3452	0.3775	0.3087	0.1545	0.1759	0.1695
YB	0.3150	0.3990	0.5326	0.3030	0.3877	0.4134	0.3940	0.4384	0.3371	0.2180	0.2417	0.2339

Appendix D. (continued)

pop ID	AH	JT	HJ	GN	HG	SM	SH	SA	AT	AB	XB	KM	YB
HT	0.7302	0.8030	0.7768	0.8040	0.8124	0.7815	0.7978	0.8152	0.7688	0.8161	0.7833	0.7499	0.7298
KT	0.6492	0.6771	0.6601	0.6782	0.6813	0.6823	0.6907	0.6854	0.6890	0.7152	0.7269	0.6973	0.6710
AG	0.6139	0.6415	0.6716	0.6895	0.7056	0.6754	0.6820	0.6947	0.6385	0.6895	0.6672	0.6376	0.5871
D1	0.7522	0.7696	0.7534	0.7706	0.7723	0.7943	0.7724	0.7774	0.7400	0.7783	0.8157	0.7871	0.7386
D2	0.7032	0.7769	0.7141	0.7315	0.7397	0.7757	0.7246	0.7656	0.6812	0.7304	0.7566	0.7277	0.6786
G3	0.6873	0.7014	0.6983	0.7164	0.7615	0.7396	0.7090	0.7271	0.6647	0.7171	0.7418	0.7120	0.6614
G4	0.6884	0.7161	0.6847	0.7123	0.7555	0.7354	0.7036	0.7215	0.6703	0.7183	0.7369	0.7081	0.6744
G9	0.6743	0.6327	0.6330	0.6440	0.6060	0.6658	0.6457	0.6207	0.6297	0.6570	0.7099	0.6856	0.6451
LY	0.7147	0.7886	0.7619	0.7896	0.8342	0.7665	0.7831	0.8008	0.7539	0.8010	0.7684	0.7344	0.7138
CA	0.9111	0.9653	0.9270	0.9472	0.9096	0.9263	0.9365	0.9256	0.8914	0.9352	0.8874	0.8569	0.8041
NJ	0.9080	0.9896	0.9235	0.9416	0.9036	0.9338	0.9302	0.9347	0.8910	0.9207	0.8746	0.8387	0.7853
ZJ	0.9402	0.9277	0.9532	0.9718	0.9344	0.9513	0.9622	0.9505	0.9250	0.9342	0.8882	0.8441	0.7915
AH	****	0.8909	0.9146	0.9346	0.8975	0.9500	0.9289	0.9133	0.9437	0.9279	0.9475	0.9054	0.8588
JT	0.1155	****	0.9089	0.9289	0.8918	0.9290	0.9134	0.9307	0.8691	0.9046	0.8555	0.8213	0.7692
HJ	0.0892	0.0955	****	0.9753	0.9448	0.9552	0.9667	0.9609	0.9219	0.9402	0.8814	0.8348	0.7852
GN	0.0676	0.0737	0.0250	****	0.9712	0.9803	0.9932	0.9873	0.9484	0.9652	0.9074	0.8606	0.8121
HG	0.1082	0.1145	0.0568	0.0292	****	0.951	0.9665	0.9786	0.9217	0.9376	0.8789	0.8309	0.7829
SM	0.0513	0.0736	0.0459	0.0199	0.0502	****	0.9731	0.9777	0.9279	0.9439	0.9224	0.8761	0.8258
SH	0.0738	0.0906	0.0338	0.0068	0.0341	0.0272	****	0.9826	0.9630	0.9693	0.9203	0.8722	0.8243
SA	0.0907	0.0718	0.0399	0.0128	0.0217	0.0226	0.0175	****	0.9376	0.9540	0.8948	0.8466	0.7985
AT	0.0580	0.1403	0.0813	0.0530	0.0816	0.0748	0.0377	0.0644	****	0.9750	0.9425	0.8961	0.8685
AB	0.0748	0.1003	0.0616	0.0354	0.0644	0.0577	0.0312	0.0471	0.0253	****	0.9350	0.8953	0.8711
XB	0.0539	0.1561	0.1263	0.0972	0.1290	0.0807	0.0830	0.1112	0.0592	0.0672	****	0.9647	0.9256
KM	0.0994	0.1968	0.1806	0.1501	0.1853	0.1323	0.1368	0.1665	0.1097	0.1106	0.0359	****	0.9730
YB	0.1522	0.2624	0.2418	0.2081	0.2448	0.1914	0.1933	0.2250	0.1410	0.1380	0.0773	0.0273	****

VITA

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